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EXAMINATION OF THE ADRENALINE INDUCED HYPERPOLARIZATION IN THE POSTGANGLIONIC NEURONES OF AMPHIBIAN SYMPATHETIC GANGLIA

by

(C)

Paul Edward Rafuse

A THESIS

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Date October 7, 1985

TO MY SISTER BETH

ABSTRACT

The hyperpolarization in response to to exogenously applied adrenaline (Ad_H) was examined in Rana pipiens and Rana catesbeiana sympathetic ganglia using the sucrose-gap and intracellular recording techniques. The investigations undertaken addressed the following considerations: 1) What is the adrenoceptor subtype which mediates the Ad_H? 2) What are the cellular events associated with the Ad_H? 3) Is all or part of the Ad_H produced by stimulation of the electrogenic NaK-pump and is orthovanadate a suitable tool to examine this possibility? 4) If the Ad_H results from a selective increase in K^+ conductance (gK), does it have a requirement for extracellular or intracellular Ca^{2+}? 5) Does adrenaline serve a neurotransmitter role in slow synaptic inhibition in amphibian ganglia?

The findings in this thesis can be itemized as follows: 1) The Ad_H appears to be mediated by an α_2-adrenoceptor. 2) Although the Ad_H has been previously reported to exhibit a slight sensitivity to the NaK-pump inhibitor, ouabain, the antagonism of the response by the K^+ channel blockers Ba^{2+} and 4-AP is in agreement with the proposal by Smith (1984a) that the Ad_H is generated by an increase in gK, instead of electrogenic NaK-pump activation. 3) Furthermore, orthovanadate was found to be a poor pharmacological tool for studying putative NaK-pump responses, since it apparently did not gain access to its cytoplasmic site of action. 4) By comparing the Ad_H with

responses which have been well established to result from an increase in gK, activated by intracellularly released Ca^{2+} (ie. the $Caff_H$'s), it was concluded that the Ad_H does not involve an identical ionic mechanism. No clear role for intracellular or extracellular Ca^{2+} could be established for this α_2-adrenoceptor mediated hyperpolarization (Ad_H). 5) Finally, on the basis of studies with selective pharmacological agents (DMI, yohimbine, Cd^{2+}), no obligatory role for adrenaline could be established in the slow i.p.s.p. recorded by means of the sucrose-gap technique from Rana pipiens sympathetic ganglia.

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LIST OF ABBREVIATIONS

α-MeNA	α-methyl noradrenaline
α-MeNA$_H$	α-methyl noradrenaline induced hyperpolarization
ACh	acetylcholine
ACh$_{AH}$	acetylcholine induced afterhyperpolarization
A9CA	anthracene-9-carboxylic acid
Ad	adrenaline
Ad$_D$	adrenaline induced depolarization
Ad$_H$	adrenaline induced hyperpolarization
AMP	adenosine monophosphate
AP	action potential
AP$_{AH}$	action potential afterhyperpolarization
4-AP	4-aminopyridine
ATP	adenosine triphosphate
Caff$_H$	caffeine induced hyperpolarization - collectively sCaff$_H$ and eCaff$_H$
sCaff$_H$	spontaneous caffeine induced hyperpolarization
eCaff$_H$	evoked caffeine induced hyperpolarization
Ca$_i^{2+}$	intracellular calcium
CLN	clonidine
CLN$_H$	clonidine induced hyperpolarization
COMT	catechol-O-methyl transferase
DA	dopamine
DA$_H$	dopamine induced hyperpolarization

DMI	desmethylimipramine
DNDS	4,4'-dinitrostilbene -2,2'-disulphonic acid
d-TC	d-tubocurarine
E_K	potassium equilibrium potential
E_M	resting membrane potential
E_{Na}	sodium equilibrium potential
e.p.s.p.	excitatory postsynaptic potential
gCl	chloride conductance
gK	potassium conductance
gNa	sodium conductance
IAS	initial axon segment
INA	isoprenaline
INA_H	isoprenaline induced hyperpolarization
i.p.s.p.	inhibitory postsynaptic potential
I-V	current-voltage
K_H	potassium activated hyperpolarization
$[K]_o$	extracellular potassium concentration
LHRH	luteinizing hormone releasing hormone
MCh	methacholine
MCh_H	methacholine induced hyperpolarization
m	metre
MΩ	megaohm
min	minute
mM	millimolar
msec	millisecond

μM	micromolar
mV	millivolt
μV	microvolt
nM	nanomolar
nA	nanoampere
NA	noradrenaline
NaK-ATPase	sodium-potassium activated adenosine triphosphatase
NaK-Pump	sodium-potassium activated pump
p.s.p	postsynaptic potentials
R_m	cell input resistance
sec	second
SIF	small intensely fluorescent
TEA	tetraethylammonium
TMB-8	8-(N,N-diethylamino) octyl 3,4,5, trimethoxybenzoate

CHAPTER I

INTRODUCTION

A. Isolated Ganglion Preparations

1. Rationales for studying autonomic ganglia

Aside from the virtues of understanding the mechanisms of ganglionic transmission *per se*, isolated sympathetic and parasympathetic (autonomic) ganglia have been widely used over the past 100 years to investigate a number of fundamental principles of neuronal function (for reviews see Skok, 1973; Blackman, 1974; Kuba and Koketsu, 1978; Dun, 1980a; McAfee, 1982). As early as 1890, the concept of chemical transmission was inferred by Langley and Dickinson by their demonstration that d-tubocurarine and nicotine could block ganglionic transmission *in vivo*. It was not until more than 50 years later that Eccles conducted the final experiments of an exhaustive study to show that this transmission was indeed chemical and not electrical (Eccles, 1943; 1944). About a decade prior to these physiological studies, Feldberg and his co-workers had performed experiments of a more pharmacological nature to show that sympathetic ganglia released acetylcholine (ACh) following preganglionic stimulation, and that this ACh activated nicotinic responses (Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1935). These demonstrations combined with the rigorous investigations by Eccles, firmly established sympathetic ganglia as a model preparation for the study of cholinergic synapses.

The popular belief that autonomic ganglia were simply synaptic relays for nerve impulses was brought into question by Laporte and

Lorente de No (1950) when they reported biphasic postsynaptic surface potentials in response to preganglionic stimulation of the turtle superior cervical ganglion. The realization that presynaptic stimulation could have both excitatory and inhibitory effects on the postsynaptic membrane has gradually lead to the alternative use of sympathetic ganglia as model systems of "intregated" synapses. Since 1950, numerous investigators have contributed to the current understanding that at least four postsynaptic potentials (p.s.p.'s) can be evoked by presynaptic stimulation (see Section A4 of Introduction). These p.s.p.'s affect the postsynaptic membrane for periods ranging from milliseconds to minutes and they either facilitate or inhibit the generation of a postsynaptic action potential over this time range. It is now widely accepted that synpatically released ACh acting on both nicotinic and muscarinic receptors can account for at least two of these p.s.p.'s (Kuba and Koketsu, 1978), while the other two p.s.p.'s may be generated by synaptically released catecholamines (Eccles and Libet, 1961), luteinizing hormone releasing hormone (LHRH; Jan, et al., 1979) and substance P (Konishi, et at., 1979a). These p.s.p.'s, and the putative transmitters proposed to induce them, will be dealt with in more detail in Section A4 of the Introduction.

In addition to intrinsic modulation of the synapses in sympathetic ganglion by the above mentioned neurotransmitters and their postsynaptic actions, a number of endogenous hormones and autocoids have been shown to exert both pre- and postsynaptic effects in sympathetic ganglia. Some of these substances include the peptides: angiotensin II (Brown, et al., 1980), bradykinin (Haefely, 1970), enkephalins (Konishi, et al., 1979b), oxytocin (Wali, 1984), vasopressin (Wali, 1984) and vasoactive intestinal peptide (Mo and Dun, 1984); and other naturally occurring compounds such as: adenosine (Henan and McAfee, 1983), GABA (Adams and Brown, 1975), 5-hydroxytryptamine (Watanabe and Koketsu, 1973), prostaglandin E_1 (Dun, 1980b) and histamine (Lindl, 1983). Conceivably, any or all of these substances could exert extrinsic influences on the physiological function of sympathetic ganglia.

Omitted from this list are the catecholamines: adrenaline (Ad), noradrenaline (NA) and dopamine (DA), which have been shown to exert both presynaptic (Christ and Nishi, 1971; Dun and Nishi, 1974; Dun and Karczmar, 1977; Kumamoto and Kuba, 1983; Kato, et al., 1985) and postsynaptic actions (Koketsu and Nakamura, 1976; Akasu and Koketsu, 1976; Brown and Caulfield, 1979; Galvan and Adams, 1982; Koketsu, et al., 1982b; 1982d; Smith, 1984). One of the postsynaptic actions: the direct hyperpolarizing response (ie. to adrenaline and dopamine in the frog sympathetic ganglion), is the subject of this thesis. The issue of whether this catecholamine-induced hyperpolarization might play a physiological role in transmission in sympathetic ganglia (Eccles and Libet, 1961) remains contentious and unresolved (Kobayashi and Tosaka, 1983; Horn and Dodd, 1983; Libet, 1985; Shinnick-Gallagher and Cole, 1985). Regardless of the outcome of this debate (and this thesis makes a contribution to it), it is clear that the sympathetic ganglion can be used as a model system to investigate adrenergic modulation of transmission and, in particular, transmission which is primarily cholinergic. Perhaps by understanding the cellular mechanisms of adrenergic effects in ganglia, more can be learned about how catecholamines produce their actions in the central nervous system and on sympathetically innervated end organs.

2. In vitro amphibian sympathetic ganglion preparations

Isolated ganglion preparations provide the investigator with a relatively simple neural system to study cellular mechanisms with much greater precision and resolution than would be possible if the ganglia were examined *in vivo*. Analysis of fundamental mechanisms may be complicated by reflexes (eg. baroreceptor reflex) and possible extrinisic hormonal regulatory systems which are operational in the intact animal. Furthermore, in *in vivo* experiments the animal itself dictates the experimental conditions to a large degree. By isolating the ganglia, the experimenter is free to alter the environment (eg. chemical composition, osmolarity, temperature) of the tissue. Also, certain electrophysiological techniques such as intracellular and sucrose-gap recording become more readily feasible using *in vitro* preparations.

The isolated paravertebral sympathetic ganglia of the frog species *Rana catesbeiana* and *Rana pipiens* offer a few practical advantages over mammalian superior cervical ganglion preparations. Although both preparations can be maintained in vitro for many hours and the mammalian ganglion may be more physiologically relevant to human considerations, the amphibian preparation is much less susceptible to physical and anoxic damage. Experiments can be successfully performed at room temperature and the more easily removed connective tissue (without damaging the neurones) permits superfused drugs better access to the ganglion cells (Nishi and Koketsu, 1960; cf. McAfee, 1982).

Another reason why the isolated amphibian sympathetic ganglion is an appropriate preparation for the studies comprising this thesis, is that much of the previous work that this thesis is based upon was performed on bullfrog sympathetic ganglia. The hypothesis of Libet and Kobayashi (1974) that an adrenergic interneurone generates the slow i.p.s.p. (see Section C of Results) and the proposal of Koketsu and Nakamura (1976) that the Ad_H results from electrogenic NaK-pumping (see Section D of Results) were both formulated on the basis of data obtained from this preparation. The examination of whether a K^+ conductance (gK), and in particular a Ca^{2+}-activated gK, is involved in the Ad_H (Smith, 1984a) is aided by prior evaluations of the various types of K^+ channels in these neurones (Adams, et al., 1982; Pennefather, et al., 1985). Finally, a drug induced Ca^{2+} activated gK, the $Caff_H$ (see section E of Results), has been well characterized in these cells (Kuba and Nishi, 1976; Kuba, 1980) which enables an evaluation of whether the Ad_H is generated by a similar ionic mechanism.

3. Anatomy and physiology of sympathetic ganglia

The early works of Bishop and Erlanger established that the amphibian paravertebral sympathetic chain is comprised of two populations of axons: rapidly conducting B fibres and slowly conducting C fibres (Bishop and Heinbecker, 1930; Erlanger and Gasser, 1930). B fibres are myelinated while C fibres are thinner in diameter and unmyelinated (Skok, 1973). These preganglionic fibres originate in spinal nerves 4-8, join the sympathetic chain via the rami communicans and terminate on the primary postganglionic cell bodies in the IXth and Xth ganglia. The B fibres are supplied by spinal nerves 4-6 and the C fibres by spinal nerves 7 and 8. These are the connections that are of importance to the investigations presented in this thesis, since electrophysiological recordings (intracellular and sucrose-gap) were only made from ganglia IX and X. A *camera lucida* drawing of the amphibian paravertebral sympathetic ganglia connections is given in Fig. 1A (Skok, 1965). The verified B and C fibre pathways in amphibian ganglia, along with the ones just described, are shown in Fig. 1B (Skok, 1973).

Previous studies have identifed B and C postganglionic neurones in amphibian sympathetic ganglia on the basis of input origin (see above), cell size and axonal conduction velocity (Dodd and Horn, 1983a). The soma of B cells are typically larger than those of C

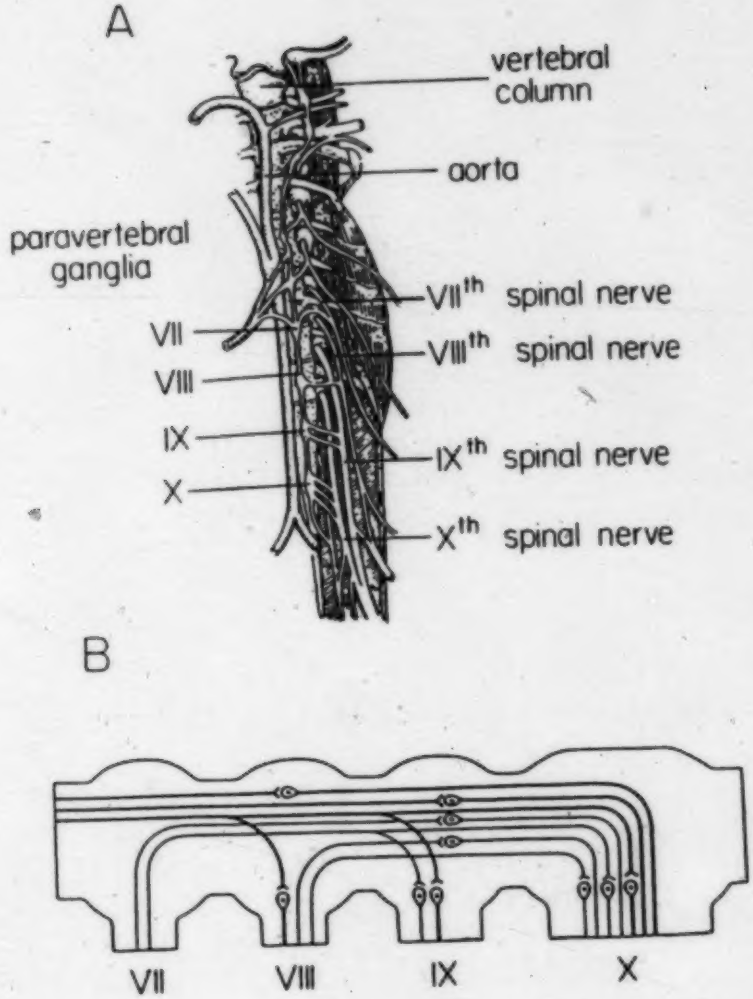

A
vertebral column
aorta
paravertebral ganglia
VIIth spinal nerve
VII
VIIIth spinal nerve
VIII
IX
IXth spinal nerve
X
Xth spinal nerve
B
VII
VIII
IX
X

Fig. 1. Anatomy and organization of frog paravertebral sympathetic ganglia. A. *Camera lucida* drawing of frog sympathetic ganglia *in situ*. Taken from Skok (1965). B. Schematic diagram of arrangment of B anc C fibres and cells in frog sympathetic ganglia. Modified from Skok (1973).

cells (Nishi, et al., 1965; Honma, 1970). Dodd and Horn (1983a) have presented photomicrographs of living bullfrog sympathetic ganglion cells, visualized by differential interference contrast optics, which show that both cell types are unipolar and ellipsoid in shape. By approximating the area of the ellipse to a circle, it was determined that the mean radius of a B cell was 25 μM and that of a C cell was 15 μM. In contrast to the amphibian cells, the several types of neurones found in mammalian ganglia are generally multipolar with branching dendrites (Skok, 1973). The careful study by Dodd and Horn (1983a) proposes that a third type of ganglionic neurone may be present in bullfrog sympathetic ganglia. Although the existence of the familiar B and C neurones with roughly matching pre- and postganglionic conduction velocities were confirmed, these authors revealed another cell type (which they referred to as a B cell subtype) with a fast conducting input (2.5 $m.sec^{-1}$), the appearance of a B soma and a slow conducting axon (0.6 $m.sec^{-1}$). Since individual cells could not be visualized in the intracellular studies of this thesis, the simpler and more established classification criteria of Nishi, et al., (1965; see Materials and Methods were used). Antidromic conduction velocities greater than 1 $m.sec^{-1}$ designated a B cell, while those less than 1 $m.sec^{-1}$ indicated a C cell.

Histochemical and electron microscopic investigations have further disclosed the presence of small intensely fluorescent (SIF) cells in the ganglia of many mammalian (Norberg, et al., 1966;

Jacobowitz, 1970; Libet and Owman, 1974; Williams, et al., 1976) and amphibian (Fujimoto, 1967; Jacobowitz, 1970; Uchizano and Ohsawa, 1973; Weight and Weitsen, 1977; Watanabe, 1980) species. It is generally agreed that SIF cells contain catecholamines since they brightly fluoresce in reaction to the Falck-Hillarp method (Falck, et al., 1962) and electron-dense granular vesicles are revealed under electron microscopic examination (Watanabe, 1983). Interestingly, while the ganglia of Rana catesbeiana stain positive for the chromaffin reaction (Weight and Weitsen, 1977), Rana pipiens ganglia stain negative (Pick, 1963).

At least four morphologically distinct granule-containing cells have been postulated to occur in sympathetic ganglia (Watanabe, 1980). Of these, it has been proposed that two functional groups of SIF cells exist (Williams, et al., 1975). According to these authors, Type I SIF cells possess long processes and lie in close apposition to primary postganglionic cell bodies. Type II SIF cells have few processes and are usually found more closely associated with blood vessels. It has been hypothesized by Libet (Eccles and Libet, 1961; Tosaka, et al., 1968; Libet, 1970; Libet and Kobayashi, 1974) and supporters (Williams, 1967; Greengard and Kebabian, 1974; Kobayashi and Tosaka, 1983) that at least some of the SIF cells (ie. Type I) in mammalian and amphibian ganglia act as adrenergic interneurones to modulate ganglionic transmission (see next Section of Introduction). It has been sugested that those SIF cells in the vicinity of blood vessels (ie. Type II) may simply serve as extra-adrenal chromaffin tissue (Weight and Weitsen, 1977).

4. Pharmacology postsynaptic potentials in sympathetic ganglia

a) Fast excitatory postsynaptic potential

It is widely recognized that the fast e.p.s.p. in both B and C cells of sympathetic ganglia is the p.s.p. resulting from presynaptically released ACh which is responsible for the transmission of an action potential through the ganglion (for review see Kuba and Koketsu, 1978). Impulses are successfully relayed across the ganglionic synapse when the amplitude of the fast e.p.s.p. is sufficient to reach the threshold for the generation of a postsynaptic action potential. This cholinergic mechanism is analogous to that described at the neuromuscular junction (for review see Steinbach and Stevens, 1976). With an overall duration of approximately 30-50 msec in bullfrog sympathetic ganglia (Weitsen and Weight, 1977), this e.p.s.p. is designated as "fast" to distinguish it from two other e.p.s.p.'s which last from seconds to minutes (slow e.p.s.p. and late slow e.p.s.p.).

The fast e.p.s.p. was first reported using intracellular recording techniques by Rosamond Eccles (1955) in the rabbit superior cervical ganglion and by Nishi and Koketsu (1960) in the bullfrog sympathetic ganglion. It was established that the fast e.p.s.p. is mediated by a nicotinic receptor when it was noted that the response was depressed by various ganglion blocking agents including d-tubocurarine, mecamylamine and hexamethonium (Blackman, et al., 1963a; Eccles, 1963). The ionic mechanism of the fast e.p.s.p. appears to be essentially the same as for the end-plate potential at the neuromuscular junction (Takeuchi and Takeuchi, 1960). The reversal potential for the fast e.p.s.p. was determined to be around -10 mV (Nishi and Koketsu, 1960; Nishi, et al., 1965) and this value could be shifted by changing the external Na^+ and K^+ concentrations (Koketsu, 1969). It has been concluded from these studies that the fast e.p.s.p. occurs as a result of increases in membrane conductances to Na^+ and K^+. A diagrammatic summary of the fast e.p.s.p. and the other p.s.p.'s to be described is given in Fig. 2 (Weight, 1983).

b) Slow excitatory postsynaptic potential

A slow excitatory postsynaptic potential (slow e.p.s.p.) can be recorded intracellularly from B cells of bullfrog sympathetic ganglia following tetanic stimulation of presynaptic B fibres in the sympathetic chain (Nishi and Koketsu, 1968b; Tosaka, et al., 1968; Weight and Votava, 1970). The slow e.p.s.p. may last from 30 to 60 seconds, making it clearly distinguishable from the fast e.p.s.p. Pharmacologically, the slow e.p.s.p. is distinct from the fast e.p.s.p. in that it is insensitive to nicotinic antagonists, but is selectively antagonized by the muscarinic blocker, atropine (Nishi and Koketsu, 1968b; Tosaka, et al., 1968). It would therefore appear likely that the slow e.p.s.p. results from the activation of postsynaptic muscarinic receptors by presynaptically released ACh.

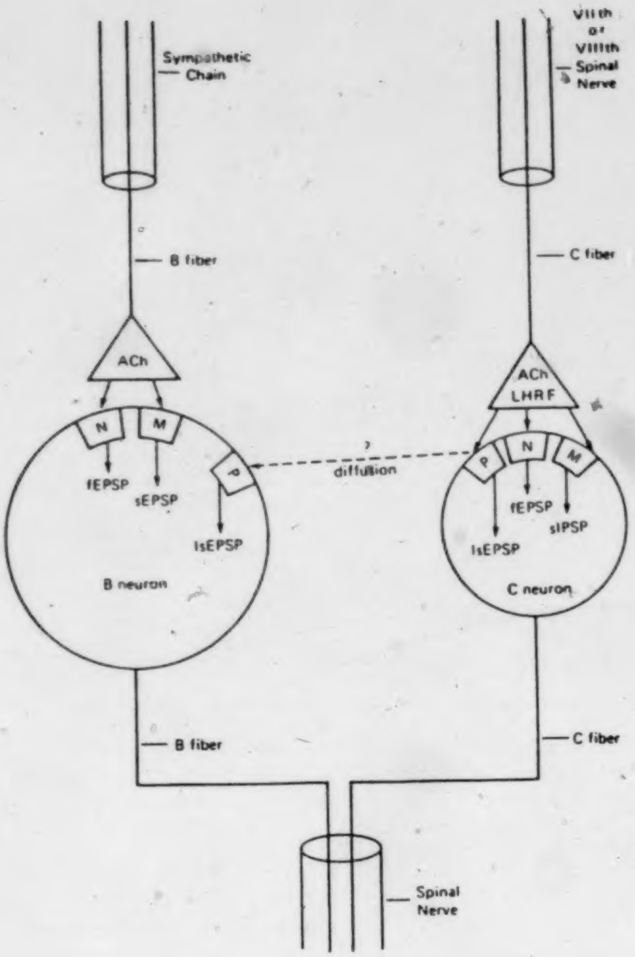

Sympathetic Chain
VIIth or VIIIth Spinal Nerve
B fiber
C fiber
ACh
ACh
LHRF
N
M
P
?
diffusion
P
N
M
fEPSP
sEPSP
lsEPSP
fEPSP
sIPSP
lsEPSP
B neuron
C neuron
B fiber
C fiber
Spinal Nerve

Fig. 2. Schematic diagram showing proposed neurotransmitters and receptors for postsynaptic potentials in B and C neurones of amphibian sympathetic ganglia. Taken from Weight (1983).

The ionic mechanism for the generation of the slow e.p.s.p. was proposed to involve a decrease in resting potassium conductance (gK) (Weight and Votava, 1970). Since 1970, this hypothesis has been substantially reinforced and elaborated by more detailed investigations using voltage-clamp techniques (Brown and Adams, 1980; Adams and Brown, 1982, Akasu and Koketsu, 1982; Jones, 1985). In particular, Brown and Adams (1980) suggested that the muscarinic action of ACh inactivated a voltage-dependent K^+ current which they called an "M-current". Further studies on mammalian ganglion cells have indicated that additional ionic events may accompany the closure of M-channels to influence the amplitude of the slow e.p.s.p. (Akasu, et al., 1984a; Brown and Selyanko, 1985; Jones, 1985). However, it remains most probable that the predominant cause of muscarinic depolarizations in sympathetic ganglia is due to M-channel closure (Brown, 1984).

The physiological purpose of the slow e.p.s.p. probably relates to its effect of enhancing ganglionic transmission for prolonged time periods (ie. tens of seconds; Brown, 1983).

c) Late-slow excitatory postsynaptic potential

Long trains of repetitive stimulation (eg. 2 sec at 50 Hz) of preganglionic C fibres in the VII or VIII spinal nerves have been reported to elicit a very long lasting e.p.s.p. which can be recorded intracellularly from both B and C neurones (Nishi and Koketsu, 1968a; Schulman and Weight, 1976; Jan, et al., 1979). Popularly referred to as the late-slow e.p.s.p., this p.s.p. may persist for as long as 5 to 10 minutes (Weight, 1983). The late-slow e.p.s.p. is unaffected by cholinergic antagonists (Nishi and Koketsu, 1968a; Jan, et al., 1979; Kuffler and Sejnowski, 1983) and in 1979, Kuffler and his colleagues presented compelling evidence that the late-slow e.p.s.p. is produced by a naturally occuring LHRH-like peptide (Jan, et al., 1979). This is an intriguing synaptic mechanism, not only because it appears to involve a unique peptidergic transmitter, but because it has recently been suggested that the LHRH-like substance released from C fibre terminals may be co-existent and co-released with the ACh in these terminals and that once released this LHRH-like substance may produce slow depolarizing responses (late-slow e.p.s.p.-like) in neighbouring B cells (Jan and Jan, 1983). It is believed that, in the absence of avid uptake or enzymatic degradation processes, this peptide may diffuse considerable distances to affect other neurones "non-synaptically".

The ionic mechanism of the late-slow e.p.s.p. has been suggested to be essentially the same as for the slow e.p.s.p. - namely suppression of the M-current (Brown and Adams, 1980; Kuffler and Sejnowski, 1983). Functionally, the late-slow e.p.s.p. may serve to enhance the facilitatory action of the slow e.p.s.p. The decrease in membrane conductance should enhance fast e.p.s.p.'s in a fashion predicted by Ohm's Law (Schulman and Weight, 1976). Furthermore, the voltage-dependence of the M-current is such that it may serve to limit repetitive discharge (Brown, 1983; Jones, 1985). Suppression of this current during the slow e.p.s.p. and late slow e.p.s.p. would therefore enhance the likelihood of cell firing.

d) Slow inhibitory postsynaptic potential

The slow inhibitory postsynaptic potential (slow i.p.s.p.) generated by either single or repetitive stimulation of the preganglionic C fibres is difficult to detect in curarized ganglia using intracellular techniques (Kuba and Koketsu, 1978). Slow positive potentials were recorded extracellularly from curarized mammalian (Eccles, 1943) and reptilian ganglia (Laporte and Lorente de No, 1950) many years before the first intracellular records of slow i.p.s.p.'s were published (Tosaka, et al., 1968). The most convenient method of recording slow i.p.s.p.'s is by the sucrose-gap technique (Kosterlitz and Wallis, 1966; Nishi and Koketsu, 1968b; Weight and Smith, 1980). Slow i.p.s.p.'s lasting between 1 to 4 seconds are typically recorded by this method.

Due to the difficulty in recording slow i.p.s.p.'s by intracellular techniques, the ionic mechanism of this p.s.p. has been difficult to determine. Weight and Padjen (1973a; 1973b) initially proposed that a decrease in resting sodium conductance (gNa) accounted for their observations on the slow i.p.s.p. in nicotinized bullfrog sympathetic ganglia. Weight has since revised this hypothesis to include a simultaneous increase in gK with the decrease in gNa (Weight, 1983). In an earlier study it was noted that the apparent reversal potential for the slow i.p.s.p. was more negative than the estimated equilibrium potential for potassium (E_K); (Weight and Smith, 1981 see also Horn and Dodd, 1981). On the basis of theoretical considerations, this reversal of the slow i.p.s.p. beyond E_K can be explained by an increase in gK and simultaneous decrease in gNa (Brown, et al., 1971). Alternatively, Nishi and Koketsu (1968b) have suggested that the slow i.p.s.p. is generated by activation of an electrogenic NaK-pump. These investigators have also re-evaluated their hypothesis and later proposed that a ouabain-insensitive portion of the slow i.p.s.p. could be explained in terms of a increase in gK (Kuba and Koketsu, 1978). Possibly the most convincing explanation of the ionic events generating the slow i.p.s.p. is provided by Horn and Dodd (1981). Aided by much better intracellular recording conditions and much larger responses (slow i.p.s.p. amplitudes as large as 40 mV), these investigators were able to show that the slow i.p.s.p. resulted from a selective increase in gK (see also Dodd and Horn, 1983b; Horn and Dodd, 1983). The same ionic mechanism has been proposed for the slow i.p.s.p. recorded from mammalian sympathetic ganglia (Cole and Shinnick-Gallagher, 1984).

The identity of the neurotransmitter and postsynaptic receptor responsible for the slow i.p.s.p. in both amphibians and mammals is currently unsettled. Two very different hypothesis have been put forward. These mechanisms are diagrammatically illustrated in Fig. 3. The monosynaptic mechanism would involve the action of

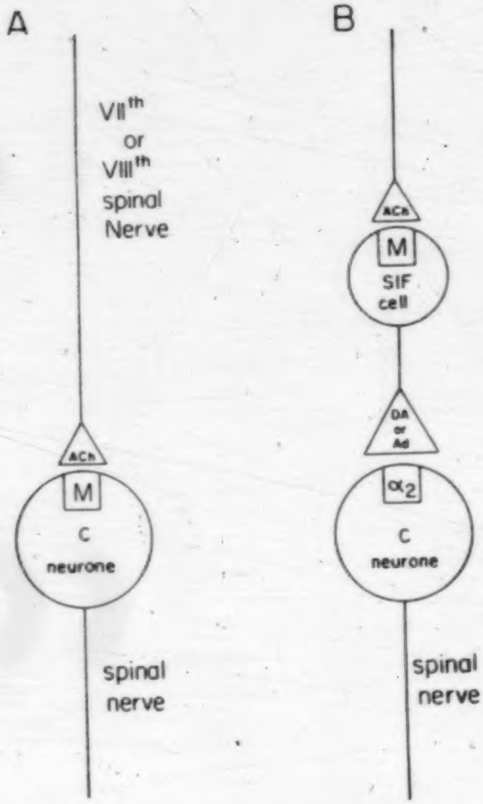

A
VII^th
or
VIII^th
spinal
Nerve
ACh
M
C
neurone
spinal
nerve
B
ACh
M
SIF
cell
DA
or
Ad
α2
C
neurone
spinal
nerve

Fig. 3. Two hypotheses proposed for the mediation of the slow i.p.s.p. in frog sympathetic ganglia. A. Monosynaptic mechanism whereby presynaptically released ACh acts directly on an inhibitory muscarinic receptor (Weight and Padjen, 1973b). B. Disynaptic mechanism whereby presynaptically released ACh acts on an excitatory muscarinic receptor residing on an interneurone which in turn releases a catecholamine (DA or Ad) onto an inhibitory α_2-adrenoceptor (Libet, 1980).

presynaptically released ACh directly on inhibitory muscarinic receptors residing on the postsynaptic neurone. The disynaptic mechanism would involve the action of presynaptically released ACh on excitatory muscarinic receptors residing on an interneurone. This muscarinic excitation would then cause the release of catecholamine from the interneurone onto an inhibitory adrenoceptor situated on the presynaptic neurone. According to the disynaptic mechanism, direct application of ACh could produce a hyperpolarizing response (during nicotinic blockade) by releasing a catecholamine from the interneurone which would act on the postsynaptic adrenoceptor.

The disynaptic hypothesis was first proposed by Eccles and Libet (1961) to explain observations made on the curarized rabbit superior cervical ganglion using extracellular recording techniques. The involvement of ACh release in the generation of the late positive potential (slow i.p.s.p.) was established by the findings that the response was abolished or depressed by botulinum toxin. The involvement of muscarinic receptors was suggested because the response was blocked by atropine. The catecholaminergic component was suggested to explain the preferential depression of the slow i.p.s.p. by the non-selective, irreversible α-antagonist, dibenamine. In addition, chromaffin-like cells were observed to be present and a previous study had shown that a catecholamine-like substance could be released from ganglia upon presynaptic stimulation (Bulbring, 1944).

The disynaptic hypothesis was suggested to apply to amphibian ganglia by analogy with the proposition for mammalian ganglia (Tosaka, et al., 1968). The first evidence in support of this hypothesis in bullfrog ganglia was provided by Libet and Kobayashi (1974). They found: (i) the slow i.p.s.p. was antagonized by the non-selective α-adrenoceptor antagonists, phentolamine (40 or 200 μM) and dihydroergotamine (40 μM), (ii) the slow i.p.s.p. was potentiated by 3',4' dihydroxy-2-methylpropriophenone (U-0521, Upjohn), an inhibitor of catechol-O-methyl transferase (COMT), (iii) the hyperpolarizing response to muscarinic agonists was reduced when transmitter release from interneurones was blocked using a low Ca^{2+}, high Mg^{2+} Ringer's solution and (iv) this response was also reduced by 40 μM dihydroergtomine and blocked by 200 μM phentolamine. It should be noted that adrenaline has been proposed to serve as the transmitter for the slow i.p.s.p. in bullfrog ganglia (Libet and Kobayashi, 1974). In contrast, it has been suggested that dopamine (DA) serves the role of transmitter in mammalian ganglia (Libet and Owman, 1974) and that it acts on an α_2-adrenoceptor and not a DA receptor (Ashe and Libet, 1982).

Additional evidence which has been used to support the disynaptic mechanism in mammals is: 1) dopamine and its synthesizing enzymes are present in ganglia (Libet and Owman, 1974); 2) exogenous dopamine produces a direct hyperpolarization which mimics the slow i.p.s.p. (Libet, 1970; Dun and Nishi, 1974); 3) dopamine fluorescence can be depleted and the slow i.p.s.p. reduced by prolonged presynaptic stimulation or exposure to bethanecol (Libet and Owman, 1974); and 4) this effect is reversed by subsequent exposure to dopamine (Libet and Owman, 1974).

The pieces of evidence in support of the monosynaptic mechanism for the generation of the slow i.p.s.p. are generally arguments against the disynaptic mechanism. Weight and Padjen (1973a; 1973b) reported that the hyperpolarizing response to ACh recorded intracellularly in nicotinized bullfrog ganglia was resistant to low Ca^{2+} Ringer's solutions. This was later confirmed using sucrose-gap recording from curarized frog ganglia (Weight and Smith, 1980). Weight and Smith (1980) further demonstrated that dihydroergotamine (10 μM) failed to antagonize the slow i.p.s.p. Other investigators using concentrations of adrenergic antagonists which would block hyperpolarizing responses to exogenous catecholamines were unable to observe antagonism of the slow i.p.s.p. in mammalian sympathetic ganglia (Dun and Karczmar, 1980; Cole and Shinnick-Gallagher, 1980; 1984). Blockade of the slow i.p.s.p. in amphibian sympathetic ganglia was non-selective (ie. concentrations of the antagonist which reduced the slow i.p.s.p. also reduced the fast e.p.s.p.; Yavari and Weight, 1981; Dodd and Horn, 1983b). Further criticisms against a dopaminergic involvement in the generation of the slow i.p.s.p. in mammalian ganglia include: i) dibenamine non-selectively affected both the slow i.p.s.p. and slow e.p.s.p. (Eccles and Libet, 1961) possibly due to its known muscarinic-blocking action (Beddoe, et al., 1971); 2) the reversible depression of the slow i.p.s.p. to prolonged exposure to bethanecol has been suggested to be due to reversible desensitization of muscarinic receptors and the presence of dopamine is not required for restoration of the response (Dun, 1980); 3) the release of ^{3}H-dopamine from sympathetic ganglia preincubated with ^{3}H-tyrosine has not been demonstrated (Noon, et al., 1975; Steinberg and Keller, 1978); 4) changes in the intensity of dopamine fluroescence did not strictly correlate with changes in the amplitude of the slow i.p.s.p. (Libet and Owman, 1974); 5) the correlation between presence of SIF cells and slow i.p.s.p. is poor - for example the guinea pig superior cervical ganglion possesses a high density of SIF cells (Williams, et al., 1976) but produces a barely detectable slow i.p.s.p. (Dun and Karczmar, 1980); conversely the SIF cell population in frog ganglion is extremely sparse (Weight and Weitsen, 1977) but the slow i.p.s.p. observed is quite substantial (Dodd and Horn, 1981; Dodd and Horn, 1983b); and 6) the functional significance of SIF cells, regarding neurotransmission, has been questioned (Weight and Weitsen, 1977; Dun, 1980). These authors offer the suggestion that SIF cells may be simply extra-adrenal chromaffin tissue.

A review of the literature reveals that the most rigorous testing of the disynaptic (catecholaminergic) hypothesis for the slow i.p.s.p. has been performed in mammalian ganglia and it appears that the weight of the available evidence is inconsistent with this mechanism. Similarly, the majority of reports on bullfrog sympathetic ganglia indicate that the slow i.p.s.p. in this species is probably mediated by a monosynaptic, inhibitory, muscarinic action of ACh

(Weight and Weitsen, 1977; Weight and Smith, 1980; Horn and Dodd, 1981; Yavari and Weight, 1981; Dodd and Horn, 1983b, Weight, 1983).

B. Effects of catecholamines in sympathetic ganglia

Catecholamines have been reported to produce both facilitatory and inhibitory effects on ganglionic transmission (for reviews see Volle, 1980; Brown and Caulfield, 1981). Inhibitory actions are more commonly observed and were first described by Marrazzi (1939). Bulbring and Burn (1942) were the first to show that low doses of adrenaline could facilitate transmission in cat superior cervical ganglia. It is now recognized that both presynaptic and postsynaptic mechanisms may contribute to adrenergic modulation of ganglionic transmission.

1. Presynaptic effects of catecholamines

Christ and Nishi (1971) demonstrated that the site of the inhibitory action of adrenaline in rabbit superior cervical ganglia was presynaptic and involved a reduction in evoked ACh release. A similar mechanism was noted for dopamine (Dun and Nishi, 1974) and noradrenaline (Dun and Karczmar, 1977). The receptor type responsible for this presynaptic effect in all cases was α- and possibly α_2-adrenergic (for discussion see Brown and Caulfied, 1981). In bullfrog sympathetic ganglia, a bimodal presynaptic response to adrenaline has been reported (Kuba, et al., 1981; Kumamoto and Kuba, 1983; Kato, et al., 1985). In these studies, acute exposure of the ganglion to adrenaline resulted in a reduction in the quantal content of the fast e.p.s.p. A sustained potentiation of transmitter (ACh) release followed removal of the adrenaline. In contrast to what has been noted in mammalian ganglia (Christ and Nishi, 1971; Brown and Caulfield, 1981), the inhibitory effect of adrenaline in bullfrog ganglia was not sensitive to changes in extracellular Ca^{2+} concentrations. This inhibitory effect appeared to be mediated by an α-adrenoceptor. The mechanism for the long-lasting potentiation of transmitter release in frog ganglion has been reported to involve β-adrenoceptors and cyclic AMP (Kuba, et al., 1981).

Brown and Dunn (1983) have suggested that β_2-adrenoceptors reside on the presynaptic terminals of rat superior cervical ganglia. Activation of these receptors by isoprenaline or salbutamol produced a depolarization in the presynaptic terminal and an increase in amplitude of the compound action potential (recorded postsynaptically), following presynaptic stimulation. It was proposed that presynaptically located β_2-adrenoceptors may largely mediate the excitatory effects of catecholamines in ganglia (see also De Groat and Volle, 1966; Haefely, 1969).

2. Postsynaptic effects of catecholamines

Five effects of catecholamines on amphibian and mammalian postganglionic neurones have been reported: 1) decrease in a voltage-dependent Ca^{2+} conductance; 2) modulation of nicotinic receptor sensitivity; 3) increase in electrogenic NaK-pumping; 4) a direct membrane depolarization; and 5) a direct membrane hyperpolarization. The acute hyperpolarizing response to catecholamines (ie. adrenaline) is the primary response examined in this thesis and the background material will be presented separately in Section C of the Introduction.

Minota and Koketsu (1977) noted that adrenaline and noradrenaline, but not isoprenaline, increased the duration of the action potential and decreased the amplitude of the spike afterhyperpolarization in bullfrog sympathetic neurones. Later, Horn and McAfee (1980) demonstrated in rat sympathetic ganglion cells, that noradrenaline reversibly inhibited three Ca^{2+}-dependent potentials: i) the shoulder on the normal action potentials; ii) the spike afterhyperpolarization; and iii) the spike recorded in the presence of TTX and TEA (Ca^{2+}-spike). The effects of noradrenaline were selectively blocked by phentolamine (10 μM) and abolished in a Ca^{2+}-free media. On the basis of these studies, it was concluded that α-agonists could block a voltage-dependent Ca^{2+} conductance in ganglion cell bodies. This has been corroborated by a voltage-clamp study by Galvan and Adams (1982). McAfee et al. (1981) have further proposed that an α_2-adrenoceptor mediates this effect.

Since cholinergic terminals in sympathetic ganglia cannot be studied directly by intracellular techniques, it has been tempting to propose that α-adrenergic inhibition of ACh release is due to blockade of Ca^{2+} channels similar to those studied in postsynaptic cell bodies (Brown and Caulfield, 1981). However, a more exhaustive comparison of the catecholamine inhibition of presynaptic transmitter release and the postsynaptic decrease in Ca^{2+} conductance is needed since it has been reported by one group using bullfrog ganglia that the presynaptic effect is not sensitive to changes in extracellular [Ca^{2+}] (Kato, et al., 1985).

Koketsu and co-workers have recently presented evidence to suggest that isoprenaline and other catecholamines can depress the sensitivity of the nicotinic receptor to ACh, possibly by a β-adrenoceptor action (Koketsu, et al., 1982d). Using an irreversible ACh-receptor antagonist, erabutoxin-b, it was determined that the catecholamines affect the ACh-receptor ion channel complex by interacting with an allosteric site (Koketsu, et al., 1982c).

Catecholamines have been proposed to stimulate electrogenic NaK-pumping in bullfrog sympathetic ganglia (Nakamura and Koketsu, 1972; Koketsu and Nakamura, 1976; Akasu and Koketsu, 1976; Smith and Dombro, 1985). In fact, it has been suggested that acute activation of the electrogenic NaK-pump may account for the direct hyperpolarizing response which catecholamines produce in these neurones (Koketsu and Nakamura, 1976). This hypothesis will be dealt with in detail in the following and subsequent sections of the Introduction. Aside from this proposal for immediate pump activation by catecholamines, Akasu and Koketsu (1976) have presented data to show that prolonged exposure to adrenaline can enhance electrogenic NaK-pumping in bullfrog sympathetic ganglion cells. In these experiments, adrenaline was noted to enhance the amplitude of the potassium activated hyperpolarization (K_H, electrogenic NaK-pump paradigm, Rang and Ritchie, 1968; see also Results, Section D) over a 35 minute period. Smith and Dombro (1985) have recently shown that this adrenaline induced potentiation of the K_H can occur within 5 minutes when the catecholamine uptake blocker, desmethylimipramine (500 nM) is present. This effect appears to be mediated by an α-receptor since it is blocked by phentolamine (Akasu and Koketsu, 1976), yohimbine and prazosin (Smith and Dombro, 1985). Interestingly, dopamine was found to have no effect on another electrogenic response to Na^+ pumping, the afterhyperpolarization following the depolarization induced by a nicotinic agonist (Brown and Caulfield, 1981; also see Results, Section D on ACh_{AH}).

Direct depolarizing responses to catecholamines have been reported by a few investigators working with mammalian (De Groat and Volle, 1966; Christ and Nishi, 1971; Brown and Dunn, 1983) and amphibian ganglia (Koketsu and Nakamura, 1976; Rafuse and Smith, 1982). De Groat and Volle (1966) suggested that the depolarizations to adrenaline, noradrenaline and isoprenaline in cat superior cervical ganglia (in vivo) were mediated by β-adrenoceptors. Brown and Dunn (1983) were later able to show that the postsynaptic depolarization to isoprenaline in isolated rat superior cervical ganglia was more sensitive to blockade by butoxamine (pA_2 = 7.36) than by practolol (pA_2 = 5.14), suggesting β_2-adrenoceptor involvement. The receptor dependence of the adrenaline induced depolarization observed in bullfrog sympathetic ganglia has not been addressed (Koketsu and Nakamura, 1976; Rafuse and Smith, 1982). The ionic mechanism of catecholamine induced depolarizations has not been addressed in either mammalian or amphibian sympathetic ganglia. In mammalian parasympathetic ganglia, it has been proposed that a catecholamine induced depolarization was produced by an α_1-adrenoceptor mediated decrease in gK (Akasu, et al., 1985).

Although a number of earlier studies have reported facilitation of ganglionic transmission by catecholamines (Bulbring and Burn, 1942; Bulbring, 1944; Malmejac, 1955; Trendelenburg, 1956), it has been difficult to conclude that this effect is due to depolarization of the postsynaptic membrane. In general, the direct depolarizing responses which have been reported have been small and infrequent. In some cases they can only be observed when an α-receptor antagonist is present (De Groat and Volle, 1966; Volle, 1980) and in some species (ie. Rana pipiens) they are never observed at all (Smith, 1984a; see Results, Section A).

C. Catecholamine induced hyperpolarizations of vertebrate neurones

Direct hyperpolarizing responses to catecholamines have been reported in a variety of amphibian and mammalian sympathetic ganglia (Lundberg, 1952; De Groat and Volle, 1966; Libet and Kobayashi, 1974; Koketsu and Nakamura, 1976; Brown and Caulfield, 1979; Smith, 1984a). Catecholamine induced hyperpolarizations (recorded intracellularly) have also been studied in a variety of peripheral and central neurones including parasympathetic ganglia (De Groat and Booth, 1980; Akasu, et al., 1985), myenteric ganglia (Hirst and Silinsky, 1975; North and Suprenant, 1985) substantia gelatinosa (North and Yoshimura, 1984), spinal motoneurones (Marshall and Engberg, 1979), locus coeruleus (Aghajanian and Vander Maelen, 1982; Williams et al., 1985), hippocampus (Segal, 1981), cerebellum (Siggins, et al., 1971), cerebral cortex (Phillis, 1977) and caudate neurones (Herrling and Hull, 1980).

1. Receptor characterization

Single unit recordings of both inhibitory and excitatory actions of catecholamines have been made in several brain areas (for review see Szabadi, 1979). This would suggest that both inhibitory and excitatory adrenoceptors exist in these areas. Efforts to characterize the receptor types for these opposing actions have yielded conflicting results. Inhibitory responses to iontophoretically applied adrenergic agonists in the cerebral cortex have been antagonized by both α- (Stone and Taylor, 1977) and β-adrenoceptor blockers (Bevan, et al., 1977). Conversely, in the same brain area, noradrenaline evoked excitations have been blocked by both α- (Bevan, et al., 1977) and β-adrenoceptor antagonists (Johnson, et al., 1969). However, the β-blocker, sotalol, has been shown to antagonize noradrenaline induced depressions of activity in cerebellum (Hoffer et al., 1971; Woodward, et al., 1974), hypothalamus (Barker, et al., 1973; Geller and Hoffer, 1977), hippocampus (Segal and Bloom, 1974) and corpus striatum (York, 1970). Taken together, Szabadi (1979) has suggested that the majority of reports on the actions of catecholamines in the CNS support the generalization that excitatory responses are mediated by α-adrenoceptors while inhibitory responses are mediated by β-adrenoceptors.

More recently, Aghajanian and his colleagues have postulated another receptor scheme based on a mixture of extracellular and intracellular in vivo experiments on the locus coeruleus and some of its projection sites (Aghajanian and Rogawski, 1983). These authors suggest that, α_1-adrenoceptors in particular may mediate noradrenaline induced excitations in dorsal lateral geniculate, dorsal raphe and facial motoneurones. Inhibitions of dorsal raphe (Freedman and Aghajanian, 1984) and sympathetic preganglionic neurones (Guyenet and Cabot, 1981) by clonidine were noted and attributed to α_2-adrenoceptor stimulation. Also, autoinhibition of the locus coeruleus by noradrenaline was determined to be mediated by an α_2-adrenoceptor (Aghajanian and Van der Maelen, 1982). The concept of α_1-adrenoceptor mediated excitations and α_2-adrenoceptor mediated inhibitions (Aghajanian and Rogawski, 1983) is consistent with the functional classification of α-adrenoceptors proposed by Berthelsen and Pettinger (1977) and the recent experimental findings in parasympathetic ganglia by Akasu et al. (1985).

The concept of α_2-adrenoceptor mediated inhibitions is further corroborated by recent studies on various in vitro neuronal preparations including: rat locus coeruleus (Williams, et al., 1985), rat substantia gelatinosa (North and Yoshimura, 1984), guinea pig myenteric ganglion (North and Suprenant, 1985), cat vesical parasympathetic ganglion (Akasu, et al., 1985), rat superior cervical ganglion (Brown and Caulfield, 1979) and frog paravertebral sympathetic ganglion (Smith and Rafuse, 1983; Smith, 1984b; see

Section B of the Results). None of the hyperpolarizing responses in these preparations were sensitive to β-adrenoceptor antagonists. This is in striking contrast to the selective antagonism by β-blockers of the inhibitory catecholamine responses in the in vivo studies reviewed by Szabadi (1979). It should be noted that the early in vivo studies performed by De Groat and Volle (1966) and Haefley (1969) on catecholamine effects on cat superior cervical ganglia described β-adrenoceptor mediated facilitation and α-adrenoceptor mediated inhibition of ganglionic transmission.

2. Hypotheses for ionic mechanism(s)

The ionic events responsible for the generation of catecholamine induced hyperpolarizations have not been definitively worked out. It is conceivable that a single common ionic mechanism does not exist for all cell types in which catecholamines produce a hyperpolarizing response since different observations have been made from different tissues (for reviews see Phillis and Wu, 1981). Several mechanisms have been suggested from intracellular studies in a variety of peripheral and central neurones.

Until recently it was commonly reported that an increase in membrane conductance could not be detected during the catecholamine induced hyperpolarization. Notably this was the case in cerebellar Purkinje cells (Siggins, et al., 1971; Hoffer, et al., 1973), spinal motoneurones (Phillis, et al., 1968; Marshall and Engberg, 1979), cerebral cortical cells (Phillis, 1977) and superior cervical ganglion cells (Kobayashi and Libet, 1970). Instead, a substantial decrease in membrane conductance was noted by Hoffer et al. (1973) and Marshall and Engberg (1979). Without demonstrating a reversal potential for the noradrenaline induced hyperpolarization in the Purkinje cells, Hoffer and his colleagues (1973) deduced that a decrease in Na^+ conductance was responsible for the response. Marshall and Engberg (1979) were able to reverse the noradrenaline induced hyperpolarization in spinal motoneurones at approximately -10 mV. They reasoned that a combined reduction of both Na^+ and K^+ conductances could account for these observations.

The lack of a clear conductance increase during the small noradrenaline induced hyperpolarization in superior cervical ganglion recorded by Kobayashi and Libet (1970); has been used by Koketsu and Nakamura (1976) to support their hypothesis that catecholamines stimulate the electrogenic NaK-pump to produce their hyperpolarizing effects. This hypothesis will be dealt with in detail in the following section of the Introduction. It will be mentioned here though that two modifications to this general hypothesis have been made. Koketsu and his associates have suggested that an increase in potassium conductance may accompany electrogenic NaK-pump activation (Kuba and Koketsu, 1978). On the other hand, Segal (1981) has postulated that the noradrenaline induced hyperpolarization in hippocampal CA1 neurones may be generated by the combined action of increased NaK-pumping and an increased gCl. Teleologically, these last two suggestions are difficult to endorse since the potential change produced by electrogenic NaK-pumping would be attenuated by a concurrent increase in membrane conductance to K^+ or Cl^-.

Recently, a few groups have shown that clear conductance increases to K^+ underly the noradrenaline induced hyperpolarization in neurones of the locus coeruleus (Egan, et al., 1983; Williams, et al., 1985) myenteric ganglia (North and Suprenant, 1985), substantia gelatinosa (North and Yoshimura, 1984) and parasympathetic ganglia (Akasu, et al., 1985). It may be significant that all of these catecholamine induced hyperpolarizations were mediated by an α_2-adrenoceptor (see next Section of Introduction). The α_2-adrenoceptor mediated hyperpolarization to adrenaline (Ad_H) in Rana Pipiens sympathetic ganglia may also be generated by an increase in gK (Smith and Rafuse, 1983; Smith, 1984a). In the latter study, the amplitude of the Ad_H recorded by the sucrose-gap technique was inversely proportional to the $[K^+]_o$ and exhibited a reversal potential similar to that for the antidromically evoked action potential afterhyperpolarization (AP_{AH}).

3. Receptor-Effector Transduction Mechanisms

a) Beta adrenoceptor mechanism

Both β_1- and β_2-adrenoceptor mediated events have been associated with increases in cyclic AMP levels in a wide variety of neuronal and non-neuronal tissues (for general review see Nathanson, 1977). The most compelling example in neuronal tissue where cyclic AMP increases have been linked to a β-adrenoceptor mediated hyperpolarization is that of the action of noradrenaline on cerebellar Purkinje cells (Siggins, et al., 1971; Bloom, et al., 1975). These authors suggested that there might be a cause-effect relationship between adenylate cyclase activity and the inhibitory actions of noradrenaline on the basis of the following observations: 1) application of cyclic AMP mimicked the hyperpolarizing and depressant effects (on discharge rate) of noradrenaline, 2) phosphodiesterase inhibitors potentiated the depressant actions of noradrenaline and 3) it had previously been reported that cyclic AMP levels increased in response to noradrenaline in cerebellar slices (Kakiuchi and Rall, 1968).

b) Alpha - 1 adrenoceptor mechanisms

Much of what is known about the cellular events following α_1-adrenoceptor activation comes from biochemical studies on non-neuronal tissues (for reviews see Exton, 1981; 1982). In such diverse tissue types as liver, smooth muscle and blowfly salivary gland, α_1-adrenoceptor stimulation results in an increase in cytosolic Ca^{2+} levels and phosphotidylinositol turnover. The phospholipase C catalyzed metabolism of phosphotidylinositol to 1,2-diacylglycerol has been proposed to mediate the mobilization of Ca^{2+} (Michell, 1979), but it has been argued by others that it is the Ca^2+ influx which causes the phosphatidylinosotol breakdown (Cockcroft, 1981). Regardless of the order in which these events occur, it is universally accepted that it is the increase in cytosolic

Ca^{2+} which is critical to the eventual α_1-adrenoceptor mediated response.

Intracellular Ca^{2+} levels could be elevated by either an increase in Ca^{2+} influx from the external environment or by mobilization of Ca^{2+} from internal stores. In the case of hepatocytes, the contribution by extracellular Ca^{2+} appears to be small and nearly all of the α_1-adrenoceptor stimulated increase in cytosolic Ca^{2+} must come from as yet unidentified intracellular storage sites (Exton, 1982). The situation in smooth muscle cells is more complex. Depending on the smooth muscle type (including intestinal muscle which relaxes to noradrenaline), a biphasic contractile response involving a transient and sustained contracture may result from α-adrenoceptor stimulation (Bolton, 1979). In many smooth muscles, the tonic contracture is abolished when Ca^{2+} is removed from the bathing medium leaving a single transient component which disappears with repeated exposure to noradrenaline. It has been postulated that the transient component is due to release of Ca^{2+} from a depletable intracellular store. The receptor dependence of the fast and slow contractures in smooth muscle has not been succinctly stated, but according to the preliminary generalization of McGrath (1982) for α_1- and α_2-adrenoceptors in vascular smooth muscle: a fast component utilizing intracellular Ca^{2+} would be linked to an α_1-adrenoceptor and a slow component dependent upon extracellular Ca^{2+} would be associated with an α_2-adrenoceptor.

It should be noted that the α_1-adrenoceptor mediated responses so far identified in neurones are excitatory (depolarizations) (Aghajanian and Rogawski, 1983; Akasu, et al., 1985). However, theoretically at least, a mechanism which causes an increase in cytosolic Ca^{2+} levels could activate a calcium-dependent potassium conductance which would hyperpolarize the neurone (Horn and McAfee, 1980). Calcium activated potassium conductance increases linked to α-adrenoceptors have been reported for smooth muscle cells (Bulbring and Tomita, 1977; Bolton, 1979) parotid gland cells (Putney, 1979), adipocytes (Exton, 1982), and hepatocytes (Jenkinson, 1985).

c) Alpha-2 adrenoceptor mechanisms

It has been shown for a wide variety of tissues that α_2-adrenoceptor stimulation leads to a decrease in cyclic AMP levels (for review see Exton, 1982). This is due to a decrease in adenylate cyclase activity rather than an increase in phosphodiesterase activity (Limbird, 1983). This relationship has not been demonstrated for brain tissue using biochemical techniques, but Bylund and U'Prichard (1983) suspect that this negative result is an artefact induced by the preparation of the membrane homogenate. In fact, Andrade and Aghajanian (1984) have shown that the clonidine induced hyperpolarization in locus coeruleus neurones was reversed by 8-bromo-cyclic AMP and dibutyryl-cyclic AMP.

Whereas there is good agreement between biochemical and physiological studies of α_1-adrenoceptor mechanisms regarding the mobilization of Ca^{2+} there is very poor agreement between these approaches concerning Ca^{2+} involvement in α_2-adrenoceptor mechanisms. Biochemical studies of α_2-adrenoceptor mechanisms have not elucidated a clear role for calcium (Exton, 1982; Limbird, 1983) yet α_2-adrenoceptor contractures in vascular smooth muscle have been shown to require an influx of extracellular calcium (Timmermans and Van Zwieten, 1981; McGrath, 1982). There are also hints that Ca^{2+} may be important in α_2-adrenoceptor actions in neuronal and specifically ganglionic tissues (Brown and Caulfield, 1979; Horn and McAfee, 1980; Morita and North, 1981; Akasu, et al., 1985).

D. Catecholamine stimulation of the electrogenic NaK-pump and NaK-ATPase in nerve and muscle membranes

1. Concept of active Na^+ and K^+ counter transport

It has long been accepted that the resting membrane potential of excitable cells (and non-excitable cells for that matter) results from the presence of ionic concentration gradients across a membrane which is primarily permeable to K^+ (Kuffler and Nicholls, 1976). The awareness that the membrane was also marginally permeable to Na^+ prompted many investigators in the first half of this century to speculate that an energy requiring mechanism might exist for transporting Na^+ and K^+ against their concentration gradients (for a historical review of the developments in excitable cells see Phillis and Wu, 1981).

Schatzmann (1953) demonstrated that the transmembrane movements of Na^+ and K^+ against their concentration gradients in red blood cells depended upon the presence of ATP and that such ion "pumping" could be inhibited by cardiac glycosides. A few years later, Skou (1957) established that a Mg^{2+} dependent ATPase activity isolated from crab nerves was regulated by Na^+ and K^+. Later, Skou (1965) proposed that this ATP hydrolyzing enzyme might provide the machinery for active transport of Na^+ and K^+. The definitive confirmation of this proposal awaited the demonstration by Hilden and Hokin (1975) whereby a purified Na^+, K^+-activated Mg^{2+}-dependent ATPase (NaK-ATPase) was inserted into liposomes and shown to exhibit active bidirectional transport of Na^+ and K^+. The physiological correlate of the NaK-ATPase is referred to as the NaK-pump in this thesis.

2. Evidence for electrogenicity

It is now generally accepted that the NaK-pump in most eukaryotic cells is electrogenic, in that it transports more Na^+ out of the cell than K^+ in (for review of evidence in excitable cells see Thomas, 1972). The extent of electrogenicity, or the ratio of Na^+ pumped out for K^+ pumped in, has proven difficult to investigate under normal resting conditions (Phillis and Wu, 1981). The basal level of NaK-pump activity is directly proportional to the $[Na]_i$ and since this is normally quite low, so is the level of pumping. By loading the cells with Na^+, the transporting characteristics of the NaK-pump can be more clearly examined. Several means of loading cells with Na^+ have been used including: high frequency stimulation (Ritchie and Straub, 1956), exposure to a NaK-pump inhibitor such as ouabain

(Thomas, 1972), direct injection of Na^+ (Kerkut and Thomas, 1965) or prolonged incubation in low $[K^+]$ (Kernan, 1962). Hyperpolarizations occur immediately following tetanic stimulation (post-tetanic hyperpolarization) and Na^+ injection whereas the NaK-pump must be stimulated acutely, usually with extracellular K^+ (although other cations can be used; Rang and Ritchie, 1968), in order to produce a hyperpolarizing response in preparations incubated in low $[K^+]_o$. Electrogenicity is assumed if the reintroduction of K^+ hyperpolarized the membrane beyond E_K (Phillis and Wu, 1981).

The method of stimulating the NaK-pump, that has been previously inhibited by a low $[K^+]$ medium, by re-introducing K^+ for short periods (ie. potassium activated hyperpolarization; K_H) has been used to study NaK-pumping in amphibian sympathetic ganglia (Akasu and Koketsu, 1976; Rafuse, et al., 1985; Smith and Dombro, 1985; also see Section D of Results). A chemical means of introducing Na^+ into sympathetic ganglion cells which has been widely used to stimulate the NaK-pump involves the use of nicotinic agonists (Volle and Hancock, 1970; Brown, et al., 1972; Lees and Wallis, 1974; Libet, et al., 1977; Smith and Weight, 1977). The depolarization induced by the nicotinic agonist (eg.'s ACh, nicotine, carbachol) increases the membrane conductance to Na^+ and K^+ (Koketsu, 1969) and the influx of Na^+ activates the NaK-pump to produce an afterhyperpolarization following the initial depolarization (ie. ACh_{AH} in the case of ACh; see Section D of Results).

It is believed that the NaK-pump in frog sympathetic ganglia is electrogenic although the precise coupling ratio of Na^+ pumped out for K^+ pumped in has not been determined (Akasu and Koketsu, 1976; Smith and Weight, 1977). In the cell types where the coupling ratio has been calculated, it covers a range from 4Na:3K in snail neurones (Thomas, 1969) to 5Na:1K in frog muscle (Keynes, 1965). Mullins and Brinley (1969) made a detailed analysis of active transport in dialyzed squid axons and found that the coupling ratio changed with the $[Na]_i$ from 1Na:1K (electroneutral) at low $[Na]_i$ to 3Na:1K at high $[Na]_i$. At physiological $[Na]_i$, the ratio was approximately 3Na:2K which is the same as that for red blood cells (Post, et al., 1967). Thomas (1972) states in his review that the coupling ratio, 3Na:2K, may be a good approximation for most excitable cells under resting conditions.

3. Physiological importance of the electrogenic NaK-pump in excitable cells

The role of the electrogenic NaK-pump in maintaining Na^+ and K^+ gradients is of universal importance to all eukaryotic cell types. It is particularly important to nerve and muscle cells. The Na^+ and K^+ gradients of these cells are diminished during prolonged periods of high frequency firing. The cells become loaded with Na^+ and depleted of K^+. As was mentioned in Section C2 of the Introduction, the elevation of $[Na]_i$ would activate the NaK-pump and this would transport the accumulated Na^+ out and the lost K^+ back into the cell.

This mechanism would also protect neighbouring cells from an external environment of depleted [Na^+] and excess [K^+].

Due to its electrogenic properties, a basal level of NaK-pump activity exerts a steady-state hyperpolarizing influence on the membrane. Further stimulation of electrogenic NaK-pumping would increase the membrane potential and make the cell less excitable (by moving the membrane potential away from the threshold for action potential generation). Conversely, inhibition of the NaK-pump for brief periods might depolarize the membrane potential and render the cell more likely to generate an action potential. Prolonged inhibition of the NaK-pump would result in a deterioration of ion gradients and a reduction in cell excitability.

It has been widely speculated that the electrogenic NaK-pump may be under hormonal or neurotransmitter control (Vizi, 1978; Phillis and Wu, 1981). Several groups have addressed the possibility that endogenous NaK-pump inhibitors exist which might serve to regulate active Na^+ and K^+ transport (Fishman, 1979; Kim and LaBella, 1981, Akagawa, 1984; Rafuse, et al., 1985). Similarly, many other laboratories have investigated the possibility that certain neurotransmitters may activate the NaK-pump. Indirectly, excitatory transmitters such as ACh (Brown, et al., 1972; Smith and Weight, 1977) and glutamate (Padjen and Smith; 1983) may stimulate the NaK-pump following their depolarizing action. Directly, catecholamines have been postulated to enhance NaK-pump activity in a variety of excitable cells (for review see Phillis and Wu, 1981; see also following sections of Introduction). Such an action has been proposed for pre- as well as postsynaptic membranes. It has been suggested that NaK-pump activity in nerve terminals under catecholaminergic control may regulate the release of neurotransmitters (Paton, et al., 1971; Vizi, 1979). Vizi and his colleagues have presented evidence to show that inhibition of NaK-pump activity enhances ACh release and stimulation of the pump reduces release.

4. Catecholamine stimulation of the electrogenic NaK-pump

a) Skeletal muscle

The most extensively studied tissue and best supported argument for electrogenic NaK-pump stimulation by catecholamines involves the effects observed on skeletal muscle (for review see Phillis and Wu, 1981). In addition to the evidence in support of this hypothesis, the case for catecholamine induced NaK-pump stimulation in skeletal muscle is made more credible since a reasonable physiological purpose has been proposed for this mechanism. It has been proposed by Clausen (1983) that catecholamine induced NaK-pump stimulation may be the primary hormonal mechanism for reducing elevated plasma levels of K^+ during exercise induced hyperkalemia.

It has been generally observed that the catecholamine induced hyperpolarization in skeletal muscle is mediated by a β-adrenoceptor (Bowman and Raper, 1965) although more recent studies with salbutamol (Clausen and Flatman, 1980) and terbutaline (McArdle and D'Alonzo,

1981) suggest that, more specifically, a β_2-adrenoceptor may be involved. The noradrenaline induced hyperpolarization in rat soleus muscle is inhibited by low $[K]_o$, Li^+, ouabain and low temperatures (Q_{10} = 3.7; Edstrom and Phillis, 1981). The catecholamine stimulated release of $^{22}Na^+$ from frog sartorius muscle was blocked by strophanthidin (Hays, et al., 1974). On the basis of Michaelis-Menten kinetics it was determined that adrenaline decreased the Km and increased the Vmax for ouabain-sensitive $^{22}Na^+$ efflux from frog skeletal muscle (Kaibra, et al., 1982).

b) Smooth Muscle

The role of electrogenic NaK-pump activation in catecholamine induced inhibitory actions (eg.'s hyperpolarizations, relaxation, decrease spontaneous spike activity) in smooth muscle remains very controversial due to numerous intracellular studies reporting clear conductance changes during catecholamine induced hyperpolarizations (for revew see Phillis and Wu, 1981). Electrogenic NaK-pump activation as an ionic mechanism for catecholamine action in smooth muscle was originally proposed by Burnstock (1958) on the basis of observations using metabolic inhibitors. Although Bulbring and her colleagues initially provided evidence to support this assertion (Bulbring, et al., 1966), she later concluded that adrenaline could invoke membrane conductance increases to both K^+ and Cl^- in taenia coli cells (Bulbring and Tomita, 1969). Torok and Vizi (1980) have shown that α_1-adrenoceptor stimulation in guinea pig taenia coli produces a combined increase in potassium conductance and activation of the electrogenic NaK-pump. Several other investigators have also indicated that α-adrenoceptor mediated hyperpolarizations in various smooth muscles are associated with increases in potassium conductance (for review see Bolton, 1979).

c) Central Neurones

There have been relatively few reports claiming that catecholamines produce their inhibitory effects in the CNS by activating the electrogenic NaK-pump. Phillis (1974) was the first to make this proposal based on the observation that noradrenaline induced inhibitions of neuronal firing rates in rat cerebral cortex were found to be ouabain sensitive. Yarbrough (1976) further demonstrated that noradrenaline inhibitions in Purkinje cells and dopamine inhibitions in caudate neurones were both antagonized by ouabain while GABA inhibitions in these two brain areas were unaffected. In a very elaborate presentation, Phillis and Wu (1981) used assorted observations made by other investigators to contend that, of the ionic mechanisms that have been considered for catecholamine inhibitions in the CNS, activation of electrogenic NaK-pumping best explains several pieces of previously unexplained data. One such piece of data from intracellular studies was the observation by many (see Phillis and Wu, 1981) that either a slight increase or no change in membrane resistance at all accompanied catecholamine induced hyperpolarizations. These findings were considered by Phillis and Wu (1981) to be consistent with the NaK-pump hypothesis for catecholamine actions. It should be noted however that several recent intracellular studies have reported substantial increases in membrane conductance during catecholamine induced hyperpolarizations in CNS neurones (Segal, 1981; Aghajanian and Vander Maelen, 1982; Egan, et al., 1983; North and Yoshimura, 1984; Williams, et al., 1985).

d) Sympathetic Ganglion cells

It has been proposed that the adrenaline induced hyperpolarization (Ad_H) in bullfrog sympathetic ganglia recorded by the sucrose-gap technique is generated by stimulation of the electrogenic NaK-pump (Nakamura and Koketsu, 1972; Koketsu and Nakamura, 1976). These authors provided the following evidence for this hypothesis: 1) the Ad_H was reduced but not eliminated by hyperpolarizing conditioning currents which were sufficient to abolish the afterhyperpolarization of the directly evoked action potential; 2) the Ad_H was reduced by either increasing (2-10 mM) or decreasing (2-0.2 mM) $[K^+]_o$, 3) the Ad_H was reduced by low $[Na^+]_o$ and eliminated in solutions in which the Na^+ had been replaced with Li^+, 4) the Ad_H was reduced by low temperatures, 5) the Ad_H was reduced by prolonged exposure to ouabain, 6) the Ad_H was not affected by replacing extracellular Cl^- with either glutamate or thiosulphate, and 7) the Ad_H was potentiated by the K^+-channel blocker TEA (5 mM). These data were interpreted as ruling out the possibilities that an increase in gK (points 1,2,7), increase in gCl (point 6) or decrease in gNa (point 3) were involved in the generation of the Ad_H. Points 1,3,4 and 5 were regarded as supportive of the hypothesis that activation of the electrogenic NaK-pump was responsible for the generation of the Ad_H.

This hypothesis has been recently re-evaluated by Smith (1984a) in frog (Rana pipiens) sympathetic ganglia using the sucrose-gap technique. While several points made by Koketsu and Nakamura (1976) were corroborated, a few results were clearly inconsistent with their NaK-pump hypothesis. Smith confirmed that the Ad_H was depressed by elevated $[K^+]_o$ but found that low $[K^+]_o$ consistently potentiated the response. These observations are opposite to what would be expected of a NaK-pump mediated response, but consistent with the concept that the Ad_H results from an increase in gK. Also, Smith was able to demonstrate reversal of the Ad_H with hyperpolarizing conditioning currents which similarly reversed the afterhyperpolarization of the directly evoked action potential. Again this suggests that an increase in gK may be involved in the generation of the Ad_H.

In the study by Smith (1984a), the sensitivity of the Ad_H to ouabain was shown to be slight by comparison to the ACh_{AH} (NaK-pump paradigm; see Section D of Results). Other catecholamine induced hyperpolarizations in mammalian sympathetic ganglia have also been shown to be resistant to NaK-pump inhibitors (Libet, et al., 1977;

Brown and Caulfield, 1981). Since Koketsu and Nakamura (1976) neglected to provide a control test for NaK-pumping (eg. ACh_{AH}) in their experiments with ouabain, the gradual reduction of the Ad_H with prolonged exposure (up to 120 min) to this NaK-pump inhibitor may simply reflect a gradual deterioration of ionic gradients.

The Ad_H in amphibian sympathetic ganglia must be further investigated in order to clarify its ionic mechanism but it would appear that some of the data provided by Smith (1984a) are more consistent with an increase in gK than an increase in NaK-pumping. Although part of this thesis (see Section D of Results) examines the effect of the NaK-pump inhibitor, orthovanadate, on the Ad_H, the last section of the Results considers the possibility that the Ad_H is generated by an increase in gK which is activated by intracellularly released Ca^{2+}.

5. Catecholamine stimulation of NaK-ATPase

a) Receptor versus non-receptor hypotheses

Since Schaefer et al. (1972) first demonstrated catecholamine enhancement of NaK-ATPase activity in subcellular fractions of rat brain, there have been numerous reports of this nature in a wide variety of neural tissues (for review see Phillis and Wu, 1981). Phillis and Wu (1981) have interpreted these reports as biochemical verifications that catecholamines stimulate the electrogenic NaK-pump to produce their inhibitory (ie. hyperpolarizing) effects. This hypothesis has been brought into question (Smith, 1984a; see also previous sections of the Introduction; Section D of Results), but it still remains that, by some mechanism, catecholamines can enhance Na^+ and K^+ dependent ATP hydrolysis in isolated neural membrane homogenates. In order to explain this phenomenon, both receptor and non-receptor mechanisms have been proposed.

Phillis and Wu have been the leading proponents of the assertion that a pharmacological receptor mediates, at least part of, the stimulatory effect of catecholamines on NaK-ATPase activity (Wu and Phillis, 1978; 1979a; 1980; Phillis and Wu, 1981). These authors contend that the following observations are consistent with this hypothesis: 1) the threshold concentration for catecholamine stimulation is often less than 1 μM, 2) catecholamine receptor antagonists can often inhibit catecholamine stimulation, 3) there appear to be some structural requirements for NaK-ATPase stimulation (eg. catechol and pyrogallol were effective while phenolic compounds were not), 4) some degree of stereospecificity was noted in that l-isomers were generally 10 x more effective than d-isomers and 5) detergent treatment often eliminated the stimulatory effect of catecholamines.

The original report of ATPase stimulation by catecholamines suggest that this effect was produced by removal of an inhibitory factor on the enzyme (Schaefer, et al., 1972). Such a mechanism would not require a pharmacologically distinct receptor. The identity of the inhibitory factor has since been postulated to be a divalent cation, such as Fe^{2+} (Schaefer, et al., 1974), Ca^{2+} (Godfraind, et al., 1974) or other metals (Hexum, 1977). Part of the evidence for these proposals is that chelating agents such as EDTA can mimic the stimulatory effect of catecholamines. It has also been suggested that catecholamines may protect the isolated enzyme from lipoperoxidative damage (Svoboda and Mosinger, 1981a; 1981b). Finally, the trace element vanadium has been implicated in catecholamine stimulation of the NaK-ATPase (Cantley, et al., 1978a; Adam-Vizi, et al., 1980). This possibility will be introduced separately in the next section of the Introduction and examined in Section D of the Results.

The weight of the evidence presently available would appear to favour the contention that catecholamines can produce their stimulatory effect on the NaK-ATPase by a non-receptor mechanism. Although the enzyme can be mildly stimulated at micromolar concentrations, an examination of the concentration-effect curves by Wu and Phillis (1980) reveals that this effect is non-saturating even at millimolar levels. Furthermore, despite extensive attempts to characterize a receptor-type for mediation of catecholamine stimulation of the NaK-ATPase, the antagonists phentolamine, propranolol and chlorpromazine have all been shown to be effective blockers of this effect (Wu and Phillis, 1979a). In the same tissue (rat cortex), even 5-HT induced stimulation could be blocked by metergoline (Wu and Phillis, 1979b). It is difficult to imagine how α, β, DA and 5-HT receptors could all reside on the same enzyme. It has been suggested that if a biogenic amine receptor does exist on isolated NaK-ATPase, then it might be some sort of "catecholic" receptor which is activated by several loosely related compounds (Wu and Phillis, 1979a).

b) Catecholamine reversal of vanadate inhibition

Although it has been known since 1965 that vanadium inhibits isolated NaK-ATPase (from rat kidney; Rifkin, 1965), it was not until 1977 that this trace element was seriously considered as a possible endogenous inhibitor of the NaK-pump (Cantley, et al., 1977). The renewed interest in vanadium was sparked by the discovery that it turned out to be the puzzling inhibitory factor which was contaminating commercially prepared ATP (Sigma Grade, derived from equine muscle) during the isolation procedure (Cantley, et al., 1977; Beauge and Glynn, 1977; Hudgins and Bond, 1977). Even before the inhibitory factor had been identified, it had been speculated that it might serve a physiological role in the regulation of the NaK-pump (Beauge and Glynn, 1977; Josephson and Cantley, 1977). The analytically determined tissue levels of vanadium were generally found to be sufficient to inhibit substantially isolated NaK-ATPase preparations. For example in mammalian brain, vanadium levels have been estimated to reach as high as 1.5 μM (Nechay, 1984) and the pentavalent oxidation state of vanadium, vanadate, has been shown to inhibit NaK-ATPase from dog brain (IC_{50}=600 nM, Nechay and Saunders, 1978) and rat cortex (IC_{50}=10 μM), Wu and Phillis, 1979c).

The inhibitory mechanism of vanadate on the NaK-ATPase is schematically depicted in Fig. 4. The molecular means by which vanadate inhibits the enzyme differs from that of ouabain in a number of fundamental ways. Inhibition by vanadate: 1) requires that vanadate bind to the cytoplasmic site of the enzyme (Beauge and DiPolo, 1979; Cantley, et al., 1978b), 2) is potentiated by K^+ (Beauge and Glynn, 1978) and other cations which will substitute for K^+ (such as Tl^+, Rb^+ NH_4^+, Bond and Hudgins, 1979; Grantham and Glynn, 1979), 3) is potentiated by Mg^{2+} (Cantley, et al., 1977) and other divalent cations (Bond and Hudgins, 1979) and 4) is antagonized by Na^+ (Nechay and Saunders, 1978). In contrast, ouabain inhibition occurs as a result of binding to the extracellular surface of the enzyme; it is antagonized by K^+; and it is facilitated by Na^+; (see Schwartz, et al., 1975). It is generally believed that vanadate produces its potent inhibitory effect on the NaK-ATPase (and its weaker effects on other phosphohydrolases) by virtue of its structural similarity to phosphate. Pentavalent vanadate can adopt a trigonal bipyramid configuration resembling the transition state of phosphate during reaction with the enzyme (Macara, 1980).

Vanadium can exist in several oxidation states depending on the pH of the environment and the presence of reactive substances. At physiological pH, the predominant oxidation state will be pentavalent vanadate (meta- or ortho-vanadate, Rubinson, 1981). However, at low pH and in the presence of a reducing agent, vanadate can be reduced to tetravalent vanadyl (Macara, 1980; Nechay, 1984). This is interesting

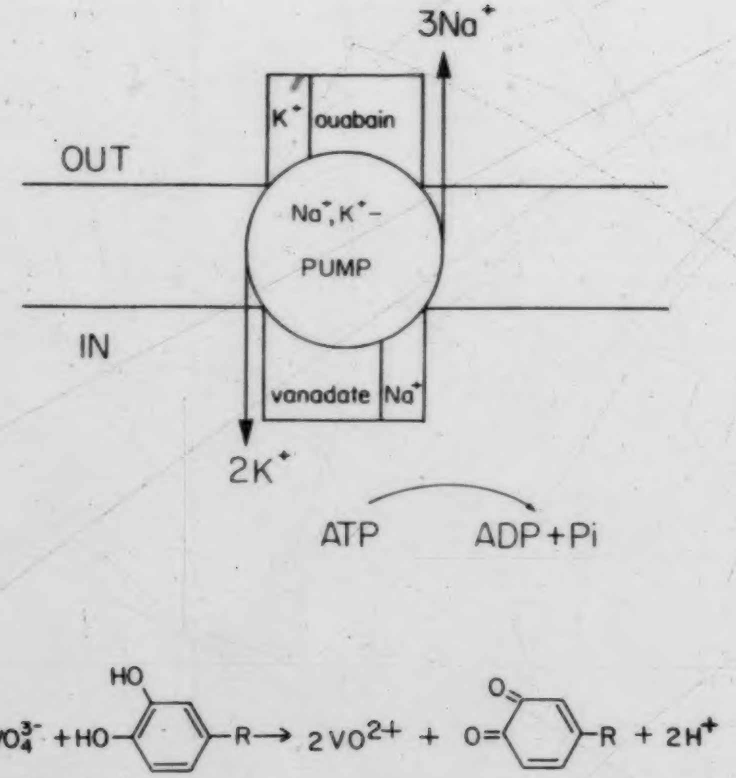

3Na⁺
K⁺
ouabain
OUT
Na⁺, K⁺-
PUMP
IN
vanadate
Na⁺
2K⁺
ATP
ADP+Pi
HO
2VO₄³⁻ +HO
R
2VO²⁺ +
O
O=
R + 2H⁺

Fig. 4. Schematic model illustrating mechanism of vanadate inhibition of the NaK-pump. Note that while ouabain acts on an extracellular site on the enzyme-pump, vanadate binds and acts at a cytoplasmic site. Catecholamines have been proposed to reverse vanadate inhibition of the NaK-ATPase by chemically inactivating the catecholamine by the formal oxidation-reduction reaction shown. Pentavalent vanadate, VO_4^{3-}, is proposed to oxidize the catecholamine to a quinone and in so doing VO_4^{3-} is reduced to inactive tetravalent vanadyl, VO^{2+} (Cantley, et al., 1978a).

since only the pentavalent forms of vanadium inhibit the Nak-ATPase; tetravalent vanadyl is apparently inactive (Cantley and Aisen, 1979; Macara, 1980). Furthermore, there are several endogenous reducing agents which could effect the conversion of vanadium from an active to inactive state including glutathione (Macara et al. 1980), ascorbate (Grantham and Glynn, 1979) and catecholamines (Cantley et al. 1978a; 1978b; Quist and Hokin, 1978; Hudgins and Bond, 1979; Wu and Phillis, 1979c). It has been proposed that catecholamines may appear to stimulate the Nak-ATPase by reversing a tonic inhibitory influence by vanadate (Cantley et al. 1978a). The suggested oxidation-reduction reaction for this mechanism is shown in Fig. 4.

Section D of the Results examines the applicability of this hypothesis to the electrogenesis of the Ad_H. If the Ad_H is produced by NaK-pump activation, this activation might occur by reversing a tonic intracellular inhibition of the NaK-pump by vanadate. Alternatively, and more simply, vanadate is used as a tool to test the hypothesis of Koketsu and Nakamura (1976) that the Ad_H is in fact the result of catecholamine induced NaK-pumping by whatever mechanism.

Electrophysiological Recording Techniques

1. Development and Applications

a) Intracellular Recording

The most direct means of monitoring the transmembrane potential of a cell is by intracellular recording methods. By positioning an electrode on either side of the membrane it is technically feasible in many vertebrate nerve and muscle cells to measure potentials (eg.'s resting, action, synaptic) which are very close to the true biological values (Fig. 5A). This was first convincingly demonstrated by Ling and Gerard (1949) when they introduced the use of fine glass micropipettes filled with concentrated KCl to impale single frog sartorius fibres. Since the 1950's, electrolyte filled pipettes with tiny tip diameters (0.5% or less of the diameter of the cell which it is to impale) have been used essentially to create a salt-bridge between the intracellular fluid and the silver wire inserted into the pipette (Purves, 1981). The electrode on the extracellular side is grounded and typically made of sintered AgCl.

Besides simply recording the membrane potential of the cell, additional glass electrodes are often used to inject current to alter the electrical gradient of the membrane or to deliver charged drug molecules (or ions) onto or into the cell. This latter technique is commonly called iontophoresis and was developed by Nastuk (1953). Constant current may be injected into a cell by various means to briefly (msec) or continuously shift the polarization of the membrane

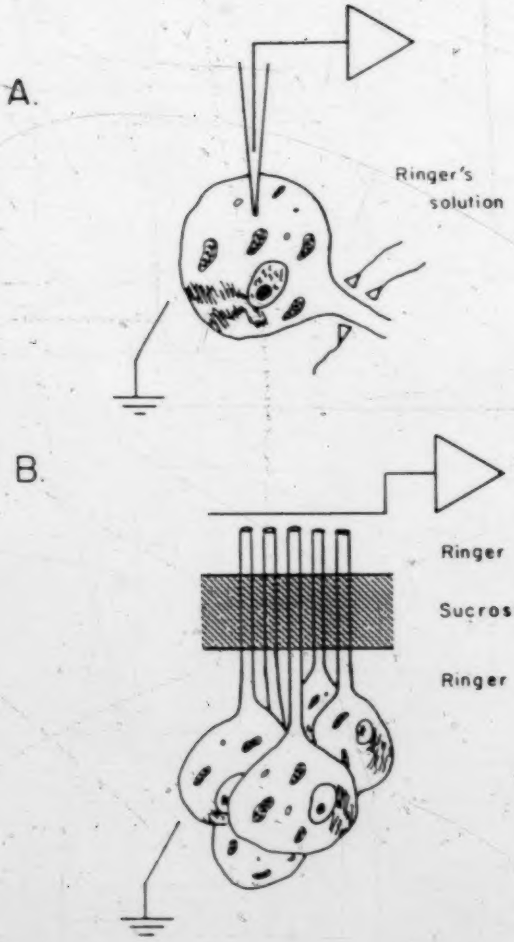

A.
Ringer's
solution
B.
Ringer
Sucrose
Ringer

Fig. 5. Schematic diagrams of A. intracellular and B. sucrose-gap recording techniques.

(Katz, 1966). Alternatively, a feedback system may be employed such that injected variable current maintains the membrane potential at a desired level. The second technique, known as voltage-clamp, was first demonstrated using two silver wires positioned in squid giant axons by Cole (1949); and his colleagues. One wire sampled the membrane potential, indicating the displacement if any from the desired voltage, while the other wire delivered the compensating current necessary to keep the membrane potential at the desired level. It later became possible to voltage-clamp vertebrate cell bodies using a single electrolyte filled glass micropipette. A further development was the introduction of the single electrode voltage-clamp technique. This involved the use of a high frequency electronic switch which rapidly switched between current injection and voltage sampling modes (Wilson and Goldner, 1975).

In the present studies, constant current could be injected through the recording electrode by utilizing a Wheatstone bridge circuit based on the procedure described by Araki and Otani (1955). This proved to be a simple but useful method to study some of the inherent membrane properties of the cells and their responses to various drugs. In particular, current injection was used to directly stimulate the postganglionic cells as well as to determine the cell input resistance by establishing a current-voltage relationship (see Materials and Methods). The voltage sensitivity and reversal potentials for drug responses were also determined by passing continuous holding currents to displace the membrane potential in either a depolarizing or hyperpolarizing direction.

b) Sucrose-Gap Recording

The technique described in these studies is representative of a general method widely used to record compound surface potentials from nerve and muscle fibres. The sucrose-gap technique is an electrophysiological method utilizing external electrodes to measure membrane potential changes between a reference and activated region of the membrane.

One of the main factors which limits the ability of surface electrodes to measure the true potential difference between active and passive membranes is the extent of short-circuiting which occurs between the electrodes through low resistance pathways in the bathing medium. This is analogous to the importance of obtaining a good seal between membrane and microelectrode in order to minimize shunting (short-circuiting) of intracellularly recorded signals (see previous section). Maximizing the resistance of the external bathing medium between the two electrodes, minimizes the short-circuiting of the membrane potential change (eg.'s drug response, synaptic potential) and therefore allows the recording of a potential change which approaches the amplitude of the true membrane event.

It was originally shown by Stampfli (1954) that by bathing the region of the membrane between the electrodes with isotonic sucrose, the electrolytes responsible for shunting the responses were washed away leaving a high resistance external environment (Fig. 5B).

The principles of the sucrose gap technique have been applied in many different instances when it is the general intention to record changes in the average membrane potential of a population of cells (neurones or muscles, for review see Wallis, et al., 1975). Some of the modifications to Stampfli's method include the use of physical barriers to strictly define the region of the preparation in contact with sucrose (Berger and Barr, 1969), the use of a double sucrose-gap (Kosterlitz and Wallis, 1964), the introduction of voltage-clamp conditions (Julian, et al., 1962a) and the simultaneous combination of sucrose-gap and intracellular recording (Julian, et al., 1962b). Imaginative chambers have also been devised to accommodate extremely short nerve branches (Dudel, 1973).

2. Comparison of the Intracellular and Sucrose-Gap Techniques

a) Experimental Indications

Slow D.C. membrane potential changes recorded intracellularly and by the sucrose-gap method will likely differ in both amplitude and time course. These dissimilarities arise from two important differences between the recording techniques. Firstly, the sucrose-gap method records from a cell population while the intracellular technique records from a single cell. Secondly, the sucrose-gap method measures potential changes along a short segment of the axons while the intracellular technique measures potential changes across a cell body membrane.

Assuming optimal recording conditions, the amplitude of the intracellularly recorded response is affected by the distance of the membrane over which the signal must travel and the geometric shape of that membrane. Electrotonic potentials are attenuated over distance (Hodgkin and Rushton, 1946). Furthermore, the cable properties of a spherical cell body are much less favourable than those of a cylindrical axon for the propagation of an electrotonic response (Jack, et al., 1975). For this reason, a small response originating in the axon or axon-hillock region may not be detected at all by an electrode remotely situated in the soma.

Another reason why it is possible that potentials recorded by the sucrose-gap technique might not be detected at all intracellularly is if the population of neurones being recorded from by the sucrose-gap method is heterogeneous. Conceivably, only one type of cell, and thus only a certain percentage of the total neurones present, is capable of responding to a particular stimulus (electrical or chemical). The sucrose-gap technique would detect the response because it is recording from all the cells, but an individually impaled neurone might not be of the necessary cell type and as a result would not respond to the same stimulus.

In the case of the sucrose-gap technique, the amplitude and duration of the population response are integrated functions of the number of cells that are activated and the synchrony in which this happens. The greater the number of neurones of the total population activated, the larger the net response. Also, as the neurones are positioned according to the anatomy of the ganglion, the signals from the activated cells will be spatially separated from one and other. As a result, the propagation distance between the signal source and point of recording will be different for each individual neurone. Therefore, even simultaneous activation of a signal would result in a recorded response which would be broader than that recorded intracellularly from a single cell. The integration of the net response is further complicated when the activation of the signal is not synchronous. It also be noted that broadening a compound response will result in a proportional reduction in the peak amplitude of the response.

Although it should be clear that many potential changes can be measured by both techniques, there are a number of indications when one method should be used and not the other. The sucrose-gap technique is chosen for studies when it is desired that average membrane potential changes are to be recorded from the entire neuronal population. This will provide an overall picture for the response to a particular stimulus. If it is suspected that a membrane potential change originates in the axon or axon-hillock area, and not in the soma, then sucrose-gap may prove to be more effective than intracellular recording in measuring these responses. For a variety of physical constraints (eg.'s extremely small cell size, presence of tough connective tissue barriers), it may be prohibitively difficult to record from single cells thus necessitating the use of the sucrose-gap method. Finally, the sucrose-gap method allows stable recording for many hours, making it suitable for pharmacological studies.

The main situation where the sucrose-gap method cannot be used and intracellular recording becomes obligatory is when it is of interest to study the biophysical events underlying a drug or transmitter induced membrane potential change in a single cell. By manipulating the electrical and chemical gradients across the soma membrane, the ionic mechanism which generates the response can be identified. The particular ion species responsible and the kinetics of its movement can only be accurately determined by using single-cell intracellular recording techniques.

b) Theoretical considerations

In order to explain the nature of the potential recorded by the sucrose-gap technique and compare it with that obtained with conventional intracellular recording, the diagrams in Fig. 6 illustrating simplified Thevenin equivalent circuits (Grob, 1984) for various parts of the postganglionic neurone will be used. A single axon model will be developed with a short discussion following on how the model is altered when recording from a population of axons. The ensuing treatment has been adapted from McAfee (1982).

The symmetrical arrangment of the apparatus in Fig. 6A establishes that the chambers on either side of the sucrose-gap are isopotential. Junction potentials at the two electrode-Ringer's

Fig. 6. A. An electrical analogue for measurements of membrane potential changes in a single postganglionic neurone by a microelectrode positioned in the soma and by the sucrose-gap method. B. Whole nerve - N axons. C. Soma and axon - single.

V - actual membrane potential change in soma

R - resistance of soma membrane

R' - resistance of leakage pathways between electrode and soma membrane

R_s - input resistance measured by intracellular electrode

E_s - membrane potential change measured in soma

R_a - resistance of pre-gap axoplasm

R_m - resistance of pre-gap axolemma

E_e - membrane potential change measured at pre-gap Ringer's solution - sucrose interface

R_A - total pre-gap resistance

R_i - internal resistance across sucrose-gap

R_g - external resistance across sucrose-gap

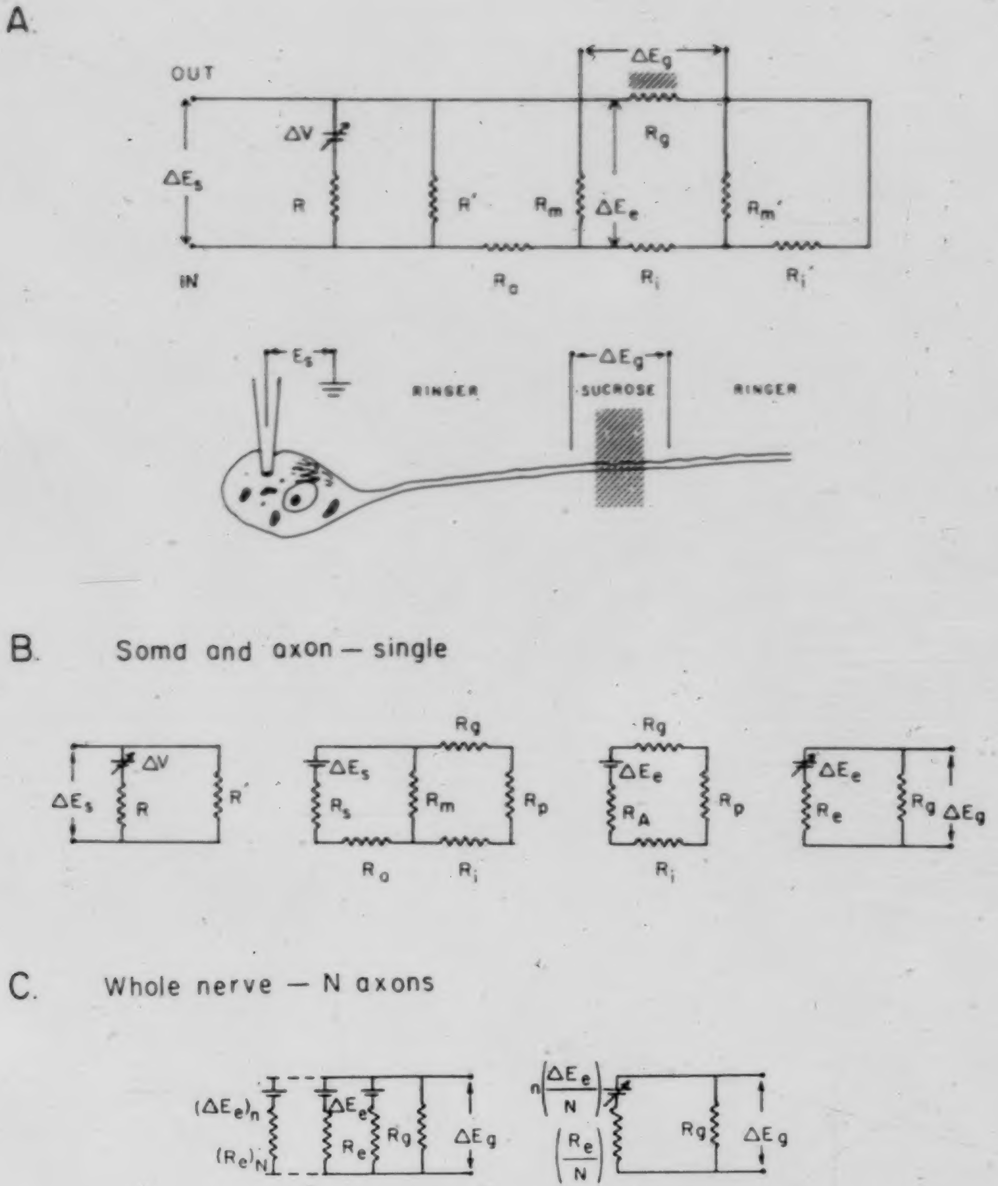
R_m' - resistance of post-gap axolemma

R_i' - resistance of post-gap axoplasm

R_p - total post-gap resistance

R_e - total electrotonic resistance minus R_g

E_g - membrane potential change measured across sucrose-gap


A.

B. Soma and axon — single

C. Whole nerve — N axons

OUT
IN
ΔE_s
ΔV
R
R'
R_m
ΔE_e
R_m'
ΔE_g
R_g
R_o
R_i
R_i'
E_s
RINGER
SUCROSE
RINGER
ΔE_s
ΔV
R
R'
ΔE_s
R_s
R_m
R_g
R_p
R_o
R_i
ΔE_e
R_A
R_g
R_p
R_i
ΔE_e
R_e
R_g
ΔE_g
$(\Delta E_e)_n$
$(R_e)_N$
ΔE_e
R_e
R_g
ΔE_g
$n\left(\frac{\Delta E_e}{N}\right)$
$\left(\frac{R_e}{N}\right)$
R_g
ΔE_g

solution interfaces and at the two Ringer's solution – sucrose interfaces will cancel out since the pre- and post-gap chambers contain solutions of identical composition. Flooding the gap with Ringer's solution or switching the positions of the electrodes will check whether the apparatus is electrically symmetrical (Wallis, et al., 1975). Neither manipulation should result in a potential change. The capacitative properties of the membrane are ignored in this treatment since only slow drug-induced or synaptic events (signals) are being considered. The signal source is taken to be the soma membrane and is indicated as a variable-voltage source (ΔV).

The network of passive conductances which make up a neuronal membrane can be simplified by reducing each point on the membrane (where it is of interest to examine the transmembrane potential) to a voltage source in series with a resistor (Thevenin equivalent circuit). At the point of intracellular recording, the measured voltage drop (E_s) occurs across the parallel resistances contributed by the soma membrane (R) and the leakage due to penetration injury (R').

$$\Delta E_s = \frac{\Delta V R'}{(R+R')} \qquad (1)$$

If penetration of the cell has produced considerable damage, R' will become very small and the input resistance (R_s) will be much smaller than the actual membrane resistance.

$$R_s = \frac{RR'}{(R+R')} \qquad (2)$$

As a result, much of the signal is shunted by the leak. Ideally, the ratio R_s/R is made as large as possible (approaching unity) by using electrodes which permit a tight seal between electrode and membrane.

$$\Delta E_s = \frac{\Delta V R_s}{R} \qquad (3)$$

As the signal (ΔV) leaves the soma and spreads electrotonically down the axon, its amplitude will diminish with distance. This is in accordance with the cable properties of a cylindrical axon such that for a given interval (eg. the pre-gap Ringer's solution-sucrose interface) the membrane potential change measured (ΔE_e) is a function of the axoplasm resistance (R_a), the axolemma resistance (R_m) and soma input resistance (R_s).

$$\Delta E_e = \frac{\Delta E_s R_m}{(R_s+R_a+R_m)} \qquad (4)$$

These three resistances can be reduced to a single value (R_A) representing the total resistance encountered between the signal source and the pre-gap Ringer's solution-sucrose interface.

$$R_A = \frac{R_m(R_s+R_a)}{(R_s+R_a+R_m)} \qquad (5)$$

The potential change measured across the gap is given by the Thevenin equivalent:

$$\Delta E_g = \frac{E_e R_g}{R_g+R_e} \qquad (6)$$

The term R_e represents the series summation of the total resistance before the gap (R_A), plus the internal resistance of the gap itself (R_i), plus the total resistance encountered after the gap (R_p).

$$R_e = R_a+R_i+R_p$$

$$R_e = \frac{(R_m)(R_s+R_a)}{(R_s+R_a+R_m)} + R_i + \frac{R_i{}'R_m{}'}{R_i{}'+R_m{}'} \qquad (7)$$

R_g indicates the external resistance of the gap which, under optimal conditions, is many times greater than R_e. As a result, ΔE_g is typically 80-95% of ΔE_e. However, if the seals at the Ringer's solution-sucrose interfaces were leaky then R_g would be decreased and the signal would be shunted.

When several axons span the sucrose-gap, the electrotonic potential carried by one axon will be shunted by adjacent axons. This means that when n axons, out of population of N axons, are conducting a potential, equation (6) becomes:

$$\Delta E_g = \frac{n(\Delta E_e/N)(R_g)}{R_g+(R_e/N)} \qquad (8)$$

In order to determine the gap potential change (ΔE_g) from a population of equivalent somatic potentials (ΔE_s), it would be necessary to know: (1) the percentage of cell bodies responding to the stimulus and (2) the distance of each individual cell body from the gap. Assuming that these pieces of information could be estimated, it is possible to calculate the amount of attenuation a signal undergoes ($\Delta E_s/\Delta E_g$) by considering the number of active cells at discrete intervals. McAfee (1982) has presented a sample calculation using rat sympathetic ganglia to show that over a distance between 50 μm and 2.5 mm (at 50 μm intervals), 100% activation of 24,000 axons results in about a nine-fold decrement between ΔE_s and ΔE_g.

F. Rationale

One of the intentions of this thesis was to characterize in some detail the sequence of membrane events which occur during an Ad_H. It was hoped that by understanding the steps involved (ie. receptor activation - transduction mechanism - transmembrane ionic movement) in this drug response, some insight might be gained into how catecholamines induce inhibitory responses in other less easily studied neuronal systems.

As a starting point in this general study, it was deemed important to establish the pharmacological identity of the adrenoceptor which mediates the Ad_H. Once it was found that the α_2-adrenoceptor antagonist yohimbine most effectively antagonized the response, this agent could also be used to study the possible involvement of Ad in the slow i.p.s.p. The nature of the ionic events accompanying the Ad_H also became more appealing once it became apparent that the adrenoceptor which mediates the response was similar to the α_2-adrenoceptor associated with presynaptic inhibition of transmitter release. This was one of the reasons for examining the participation of Ca^{2+} in the Ad_H. Since presynaptic nerve terminals cannot be directly studied in the vertebrate nervous system, it was hoped that a good understanding of postganglionic α_2-adrenoceptor function would suggest how presynaptic α_2-adrenoceptors inhibit transmitter release. Extrapolations of this nature rely on the assumption that there is a functional basis of receptor classification. By establishing the membrane events following α_2-adrenoceptor activation in amphibian sympathetic ganglia, a contribution would be made to this general issue. The veracity of this generalization can only be tested by thorough examinations and comparisons of receptor mediated events at the membrane level in a variety of tissues.

Two other aspects of the Ad_H which were scrutinized in this thesis were the hypotheses: 1) that the Ad_H resulted from stimulation of the electrogenic NaK-pump (Koketsu and Nakamura, 1976) and 2) that adrenaline released from an interneurone was the transmitter responsible for the slow i.p.s.p. (Libet and Kobayashi, 1974). The first hypothesis was investigated using the NaK-pump inhibitor orthovanadate as a tool. An ancillary aspect of this hypothesis was the possibility that the Ad_H might involve catecholamine reversal of a tonic inhibition by endogenous orthovanadate (Cantley, et al., 1978a). The second hypothesis concerning a physiological role for adrenaline was addressed since much of the evidence in support of this idea in both amphibian and mammalian ganglia was regarded as weak. The present study utilized pharmacological agents with more selective actions in an effort to clarify whether such a transmitter mechanism applies to this sympathetic ganglion (Rana pipiens).

CHAPTER II

MATERIALS AND METHODS

A. Electrophysiological preparations

1) Experimental animals

Bullfrogs (Rana catesbeiana) and leopard frogs (Rana pipiens) of both sexes were purchased from a biological supply house (Anilab, Ste. Foy, Quebec) and stored in running tap water at room temperature. Both species could be maintained in a healthy condition for up to four weeks without feeding or use of antibiotics. Bullfrogs (10-15 cm in body length) were used for the intracellular studies as well as for those sucrose gap experiments in which the actions of potassium channel blockers were examined. The remainder of the sucrose gap experiments were performed on large (at least 6 cm in body length) leopard frogs unless otherwise specified. Only the largest available leopard frogs could be used, since the sucrose gap recording chamber utilized in these experiments was originally designed to accommodate the larger ganglionic structures of the bullfrog.

2) Dissections

a) Sucrose-gap preparation

Pithed animals (bullfrogs or leopard frogs) were pinned to a dissecting tray in the supine position. The skin and the rectus abdominis were removed to expose the thoracic and abdominal cavities. The animal was then carefully eviscerated paying particular attention not to damage the sympathetic nerves lying immediately beneath the kidneys and aorta. The kidneys were removed under a dissecting microscope (Wild M3) while the aorta was left in place. Taking

Table 1

Normal Frog Ganglion Ringer's Solution

Constituents	Concentration (mM)
NaCl	100
KCl	2
$CaCl_2$ $2H_2O$	1.8
Tris HCl pH 7.2	16
D-glucose	5.6

Table 2

Solutions for Enzyme Isolation

Solution	Constituents	Concentration (mM)
Homogenizing Medium	Sucrose	250
	histidine pH 6.8	30
	EDTA (Na_2)	5
Washing Medium	EDTA (Na_2)	1
	TRIS (basic)	20
Suspending Solution	Sucrose	275
	EDTA (Na_2)	1
	TRIS (basic)	20

Table 3

Reaction Mixture for Spectrophotometric Assays

Constituents	Concentration (mM)	Activity (units[a])
glycylglycine	100	
$MgSO_4$ $7H_2O$	2	
H_4 - EDTA	0.2	
KCl	20	
NaCl	80	
Sucrose	250	
phospho(enol)pyruvate	21.8	
NADH	2.3	
laktate dehydrogenase		39.3
pyruvate kinase		62.

a = one unit converts 1.0 μmole substrate to product per min at pH 7.6 at 37°C.

special care to manipulate only connective tissue with fine forceps, the VIIth to Xth paravertebral sympathetic ganglia together with the rostral portion of the VIIIth spinal nerve, the sympathetic chain and a short portion of the IXth and Xth spinal nerve were removed (see Fig. 7) and placed in a petri dish containing chilled Ringer's solution (see Table 1). Under 40X's magnification, excess connective tissue was carefully removed from the ganglion from which recordings would be made. This was usually the ganglion (either IXth or Xth) with the longest and thickest ramus communicans. The other structures not required were trimmed away to render a preparation similar to the one illustrated in either Fig. 7A or 7B. The delicate sympathetic chain was left with some connective tissue attached for protection. At this point the preparation was ready for transfer to the sucrose gap recording chamber (see Section B1).

b) Intracellular preparation

Bullfrogs were pithed and dissected in the same way as for the sucrose gap preparation except that the VIIIth spinal nerve was not required and the IXth and Xth spinal nerves were cut as far caudally as possible (Fig. 8). Recordings were usually made from the larger of the IXth or Xth ganglion. Again, as with the sucrose gap dissection, as much of the connective sheath around the ganglion to be recorded from was removed without damaging the nerve cells. Once the preparation was transferred to the intracellular recording chamber (see Section C1; Fig. 9), it was often exposed to 1% Trypsin (EC

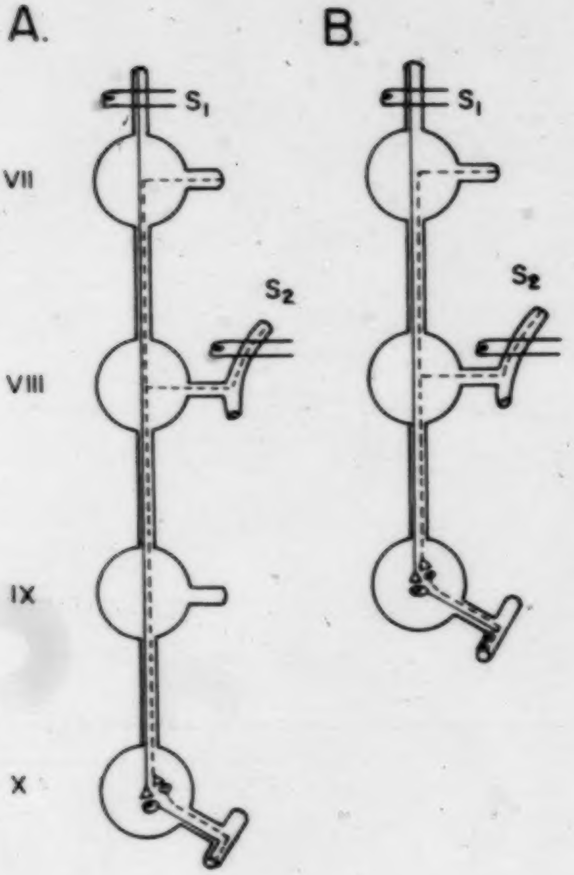


Fig. 7. Schematic drawing of *Rana pipiens* or *Rana catesbeiana* sympathetic ganglion preparations used for sucrose-gap experiments. A. Arrangment for recording from the Xth ganglion. B. Arrangement for recording IXth ganglion. S_1 indicates bipolar electrodes used to orthodromically stimulate B fibres in the sympathetic chain; S_2 indicates bipolar electrodes used to orthodromically stimulate C fibres in the rostral portion of the VIII spinal nerve. Solid lines indicate B fibres; broken lines indicate C fibres.

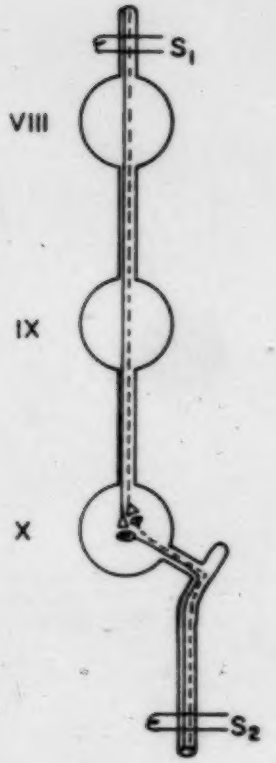


Fig. 8. Schematic drawing of *Rana catesbeiana* sympathetic ganglion preparation used for intracellular experiments. S_1 indicates suction electrode for orthodromic stimulation of B and C fibres; S_2 indicates suction electrode for antidromic stimulation of B and C cell axons. Solid lines indicate B fibres; broken lines indicate C fibres.

The illustration in Fig. 9A and the schematic diagram in Fig. 9B show the Xth ramus communicans positioned across the sucrose gap with the attached Xth spinal nerve stump placed in the right-hand (post-gap) compartment filled with Ringer's solution. With the preparation arranged in this way, net changes in the average membrane potential of the Xth ganglion cells (population potential) could be measured across the sucrose gap. The left-hand (pre-gap) compartment containing the ganglion was grounded via a Ringer-agar bridge and calomel electrode (see Fig. 9B). The right-hand compartment containing the severed axons of the ganglion cells (in spinal nerve stump) was connected (again using a Ringer-agar bridge and calomel electrode) to the amplification and recording system described in Section B3.

2) Stimulation

The sympathetic chain and the rostral portion of the VIIIth spinal nerve were positioned over bipolar platinum stimulating electrodes immersed in mineral oil as illustrated in Fig. 9B. Fig. 1B schematically illustrates the localization of two different neuronal pathways in each of these preganglionic structures. B cells could be selectively stimulated through the sympathetic chain rostral to the VIIth ganglion and C cells could be selectively stimulated through the rostral portion of the VIIIth spinal nerve (Nishi et al., 1965). A single pulse of 0.5 millisecond duration applied to the sympathetic chain was used to elicit a fast conducting B spike. A 1.0 msec pulse applied to the VIIIth spinal nerve was necessary to evoke a slowly

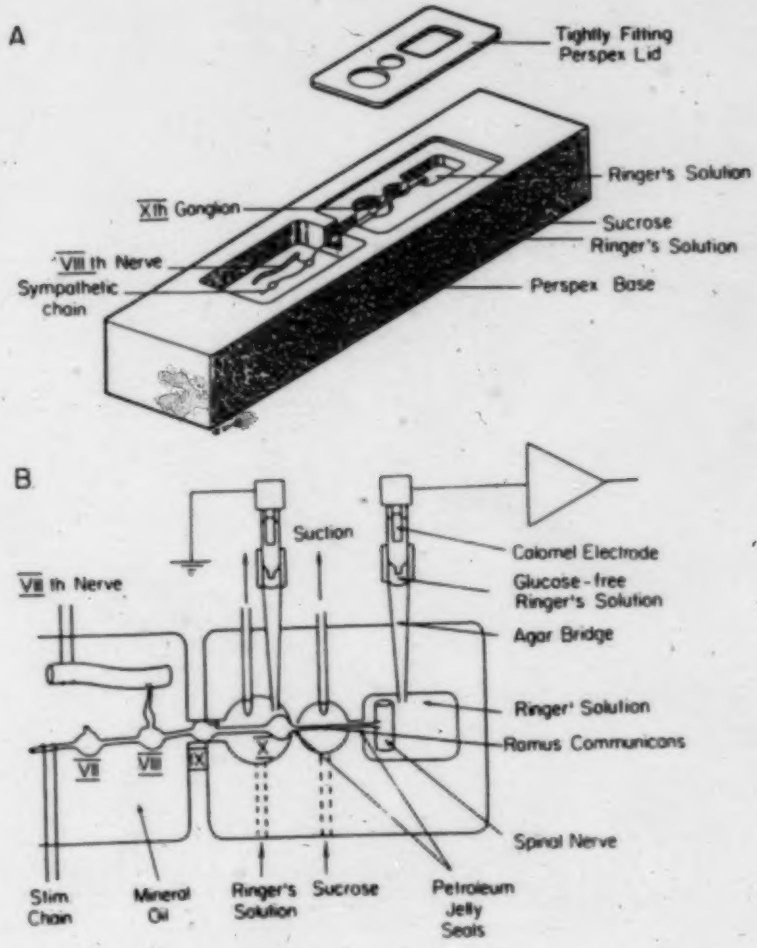

A
Tightly Fitting Perspex Lid
Ringer's Solution
XIth Ganglion
Sucrose
Ringer's Solution
VIII th Nerve
Sympathetic chain
Perspex Base
B
Suction
Calomel Electrode
Glucose-free Ringer's Solution
VIII th Nerve
Agar Bridge
Ringer' Solution
Ramus Communicans
VII
VIII
IX
X
Spinal Nerve
Stim Chain
Mineral Oil
Ringer's Solution
Sucrose
Petroleum Jelly Seals

Fig. 9. Diagrams of sucrose-gap chamber. A. Illustration of actual sucrose-gap chamber used. B. Diagram of ganglion preparation positioned in chamber with electrodes in place for stimulation of the VIIIth spinal nerve or sympathetic chain and recording from the Xth ganglion.

propagating C spike. All single pulse stimulations were delivered by Digitimer DS2 Isolated Stimulators. In order to recruit as many neurones of each cell type as possible, supramaximal stimulation was set at 1.3X's the voltage required to produce the maximal population spike size.

In the presence of 70 μM d-tubocurarine (to reduce the amplitude of the nicotinic C fibre e.p.s.p., so that it did not initiate an action potential), slow inhibitory postsynaptic potentials (slow i.p.s.p.'s) could be generated by either single shock or tetanic stimulation of the rostral portion of the VIIIth spinal nerve. Larger, more consistent responses were produced by repetitive stimulation (10 - 50 Hz) and, unless otherwise specified, the optimal stimulation parameters for slow i.p.s.p. generation were 1 msec pulses at 10 Hz for 1 second. This stimulus was delivered by connecting three stimulators where a Grass S4 gated a Grass SD9 to deliver a 1 sec train of pulses at 10 Hz with pulse lengths and intensities set by a Digitimer DS2 Isolated Stimulator.

3) Recording and display

The potential across the sucrose gap was measured using Fischer Calomel Reference Electrodes E-6A (miniature porous plug type, Cat. No. 13-639-79) as illustrated in Fig. 9B. These electrodes were fitted with Pasteur pipettes filled with 2.5% agar to form a bridge between the Ringer's solution surrounding the nerve preparation and the glucose-free Ringer's solution making contact with the porous plug of the electrode. The 2.5% agar was made up in glucose-free Ringer's solution. As indicated in Fig. 9B, the pre-gap ganglion chamber was grounded while the electrode positioned in the right-hand compartment containing the post-ganglionic fibers was connected to a high impedence amplifier. A block diagram of the stimulating and recording apparatus used for the sucrose gap studies is illustrated in Fig. 10. Recorded signals were displayed on either a Tektronix Type 564B Storage Oscilloscope or a Gould 2400 rectilinear pen recorder. The oscilloscope was used to establish the viability of the preparation by noting the amplitudes, stimulating thresholds and maximal stimulus intensities for the orthodromically generated B and C action potentials. Drug induced potential changes were displayed on the pen recorder with the frequency response adjusted from D.C. to 5 Hz. Downward deflections on the records presented indicate membrane hyperpolarization. Tetanically evoked slow i.p.s.p.'s were also displayed on the pen recorder and, unless otherwise stipulated, the frequency response was adjusted from D.C. to 15 Hz.

4) Addition of drugs

All drugs and chemicals were applied to the ganglion by superfusion. For the preparation of these solutions see Section D. Four 3-way Luer Valves (1 mm bore, Radnotti Glass, Cat. No. 120720-10) were installed as close to the inlet port of the ganglion chamber as possible. This enabled rapid and discrete changes in Ringer's solutions with minimal dead space and diffusion time without inter-

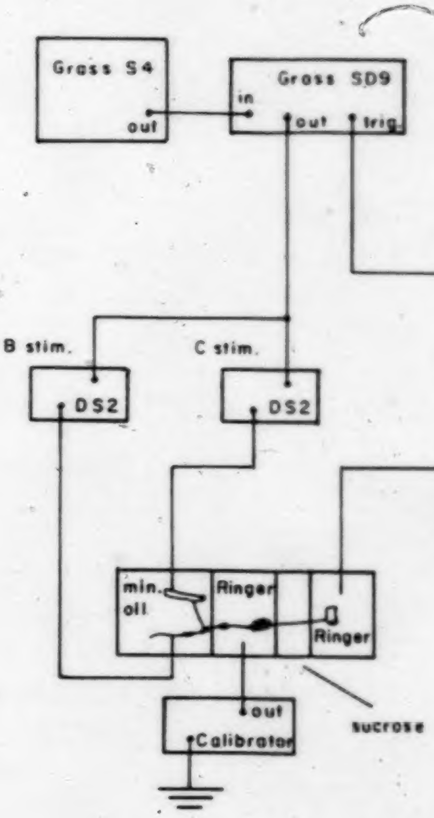

Grass S4
out
Grass SD9
in
out
trig.
Tektronix
5648
in
in
Gould 2400
V
V
low gain
in
high gain
in.
B stim.
C stim.
DS2
DS2
2·5X
min. oil
Ringer
Ringer
sucrose
out
Calibrator

Fig. 10. Block diagram of electronic apparatus used for the sucrose-gap experiments.

rupting the superfusion. With a fast flow rate of 3 - 6 ml. min^{-1}, the dead time was reduced to as little as 10 seconds. The flow rate of the Ringer's solutions (containing various drugs) was regulated by a needle valve tubing clamp positioned downstream of the 3-way valves. The Ringer's solution superfused over the ganglion was taken away by a vacuum line (Fig. 9B). Isotonic sucrose superfused over the ramus communicans was likewise removed by aspiration. Ringer's solutions and the isotonic sucrose were contained in separate reservoir bottles with rubber tubing outlets fitted near the bottom. The solutions were delivered to the 3-way valve system by polyvinyl chloride intravenous drip assemblies (Travenol JC0002). These assemblies were equipped with tubing clamps which were used to set the flow rate of the isotonic sucrose at 0.2 - 0.4 ml. min^{-1}. All superfused solutions were at room temperature (20 - 24°C).

C. Intracellular experiments

1) Chamber and preparation

The bath used for the intracellular experiments together with a sympathetic ganglion preparation is illustrated in Fig. 11. The plastic walled chamber was permanently mounted to the stage of a Wild M3 dissecting microscope which was in turn affixed to a heavy cast iron platform supported by six tennis balls which served to limit vibrations. The transparent chamber was illuminated from beneath the microscope stage by a 12 volt D.C. incandescent bulb. The entire apparatus was enclosed in a grounded Faraday Cage. The base of the

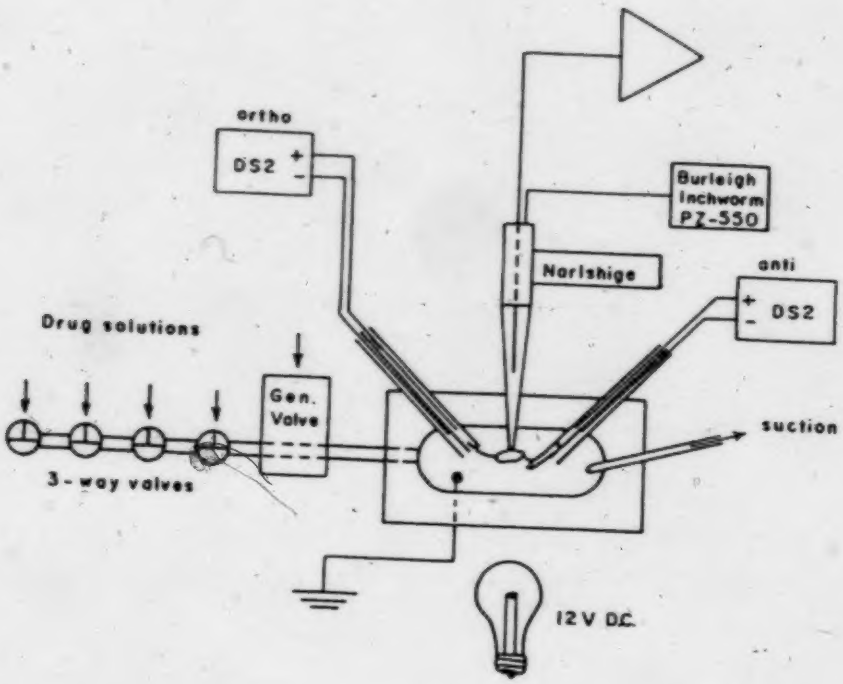

ortho
DS2
+
-
Burleigh
Inchworm
PZ-550
Narishige
anti
DS2
+
-
Drug solutions
Gen.
Valve
3-way valves
suction
12V D.C.

Fig. 11. Diagram of chamber used for intracellular experiments.

chamber was coated with translucent silicone rubber (Sylgard 1982, Dow Corning Corp.) so that the ganglion could be immobilized on the bottom of the bath with fine stainless steel insect pins (Minuten Pins, Fine Science Tools). Also embedded in the silicone base was a sintered Ag/AgCl ground electrode (Transidyne Instruments). The total volume of the bath was approximately 5 ml, but the level of superfusing Ringer's solution was usually maintained as low as possible so that the actual fluid volume was only about 1 to 2 ml.

2) Stimuation

Several suction electrodes of various aperature diameters were fashioned from drawn capillary tubing. In this way, a pipette tip could be chosen which would tightly fit the nerve to be stimulated. The ganglion was stimulated orthodromically (presynaptically) through the rostral portion of the sympathetic chain (containing B fibres) and antidromically (postsynaptically) through the caudal end of the spinal nerve (containing B and C fibres) as shown in Fig. 11. The positive terminals of the stimulating wires were placed in the glass pipettes containing the nerves and single pulses (1 msec) of at least 1.3X's maximum intensity were delivered by Digitimer DS2 Isolated Stimulators. Cell types (B or C) were identified according to the calculated conduction velocity of the antidromically evoked action potential. The criteria of Nishi, et al. (1965) were used where conduction velocities greater or less than 1 msec^{-1} designated B and C neurones respectively. Conduction velocities were calculated by dividing the distance (m) between the stimulating and recording electrodes by the latency (sec) between the stimulus artefact and the onset of the action potential.

Action potentials were also evoked by direct stimulation. This was achieved by either passing a brief depolarizing pulse of current through the recording electrode (see Section C3a) or by passing hyperpolarizing current to generate an anodal break spike.

3) Recording

a) Electrodes

Glass micropipettes were pulled on a horizontal Frederick Haer Ultrafine Micropiette Puller. A variety of capillary tubing was used but the best results were obtained with either World Precision Instruments Kwik-Fil Glass Capillaries (O.D. 1.2 mm, I.D. 0.68 mm; Cat. No. 1B120F) or Frederick Haer borosilicate capillaries with Omega Dot for rapid fill (O.D. 1.2 mm, I.D. 0.6 mm; Cat. No. 30-31-1). Successful intracellular recordings were obtained with electrodes having tip resistances ranging from 20 to approximately 150 MΩ. However, micropipettes with the sharpest tips (to minimize penetration injury) yet the lowest resistances (to facilitate current passage) were found to be optimal. Micropipettes with tip resistances between 20 and 30Ω with an approximate taper length of 10 mm were pulled with the Frederick Haer glass on the Ultrafine Micropipette Puller with the following settings (in arbitrary units): optical switch scale 105, glass preheat 225, primary pull 298, secondary pull 336, pull delay

223 and coil temperature 348. A coil configuration of seven turns with 0.014" platinum wire was used. The micropipettes were filled using a syringe containing filtered 3M KCl (Millipore, 0.45 μm pore size). Micropipettes were completely filled to the tip within twenty minutes.

The micropipette was positioned in the vicinity (within 1 mm) of the ganglion cells with a Narishige manual micromanipulator (Cat. No. 10630) which was firmly bolted to a cushioned iron platform (see Section C1). A chloride-coated silver wire was inserted in the KCl filled micropipette to connect it with the amplification and recording system diagrammed in Fig. 12. The silver wire was "coated with chloride" by connecting the wire to the positive terminal (anode) of a D.C. power source, connecting another silver wire to the negative terminal and immersing both wires in concentrated HCl for 10 - 30 sec until the wire was uniformly covered with a thin grey coating. This procedure yields a reversible electrode which will allow stable recording for long periods. The reversible redox reaction for these electrodes is $Ag + Cl^- \rightarrow AgCl + e^-$.

The steady liquid-junction potential between the 3M KCl in the micropipette and the Cl^- concentration in the extracellular Ringer's solution was compensated using the D.C. offset control in the preamplifier (Dagan 8100). This control was not altered further once a cell had been impaled since it is not known with any accuracy what the Cl^- concentration is inside these ganglion cells. It is also not

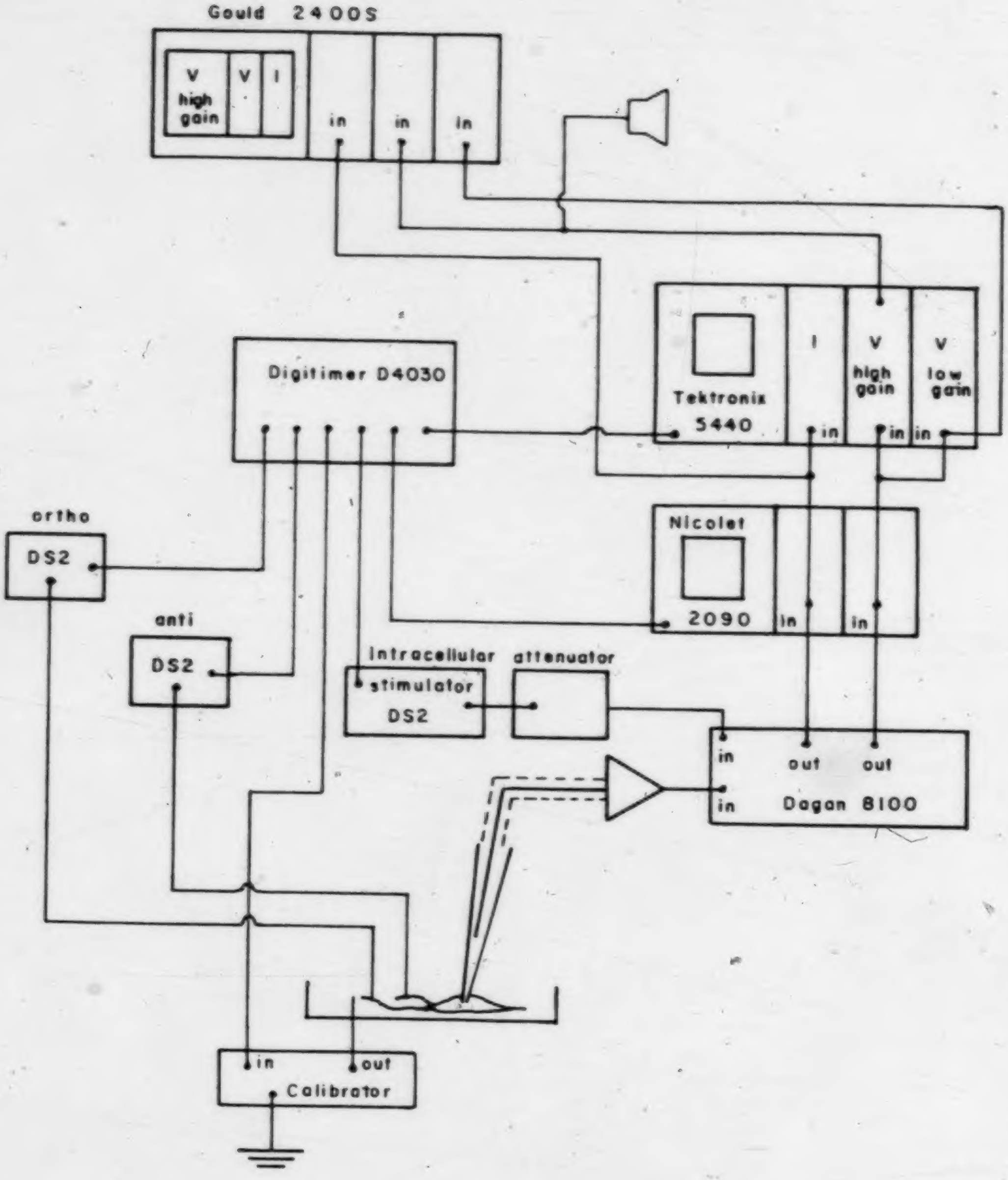

Gould 2400S
V high gain
V
I
in
in
in
Digitimer D4030
Tektronix 5440
I
V high gain
V low gain
in
in
in
ortho
DS2
Nicolet
2090
in
in
anti
DS2
intracellular stimulator DS2
attenuator
in
out
out
in
Dagan 8100
in
out
Calibrator

Fig. 12. Block diagram of electronic apparatus used for intracellular experiments.

known to what extent the electrode tip breaks upon entering the cell. Both of these factors would contribute to a "tip potential" with an unknown value. This degree of uncertainty renders measurements of absolute membrane potential slightly inaccurate (see Results).

b) Cell penetration

Before the ganglion cell was impaled, the resistance of the electrode was roughly balanced using the bridge control in the preamplifier of the Dagan 8100. This was done by adjusting the bridge control until injected current no longer produced a voltage response. Accurate balancing of the electrode resistance was only attempted once the electrode tip was firmly sealed inside the cell according to the method of Engel et al. (1972). Essentially, this method involves adjusting the bridge control until the membrane charges smoothly from the resting potential. The bridge circuit is drawn showing its likeness to the circuit devised by Wheatstone to measure resistances (Fig. 13A) and again to show its likeness to the modern circuits of an operational amplifier (Fig. 13B). An example of a membrane charging in response to a hyperpolarizing current pulse when the bridge was in and out of balanace is illustrated in Fig. 14.

After the electrode was positioned within 1 mm of the ganglion surface with the manual manipulator, a Burleigh Inchworm PZ-550 was used to slowly advance (2 μm/sec) the electrode towards the cell. It should be noted that although the Wild M3 dissecting microscope permitted clear visualization of the ganglion at 40X's magnification,

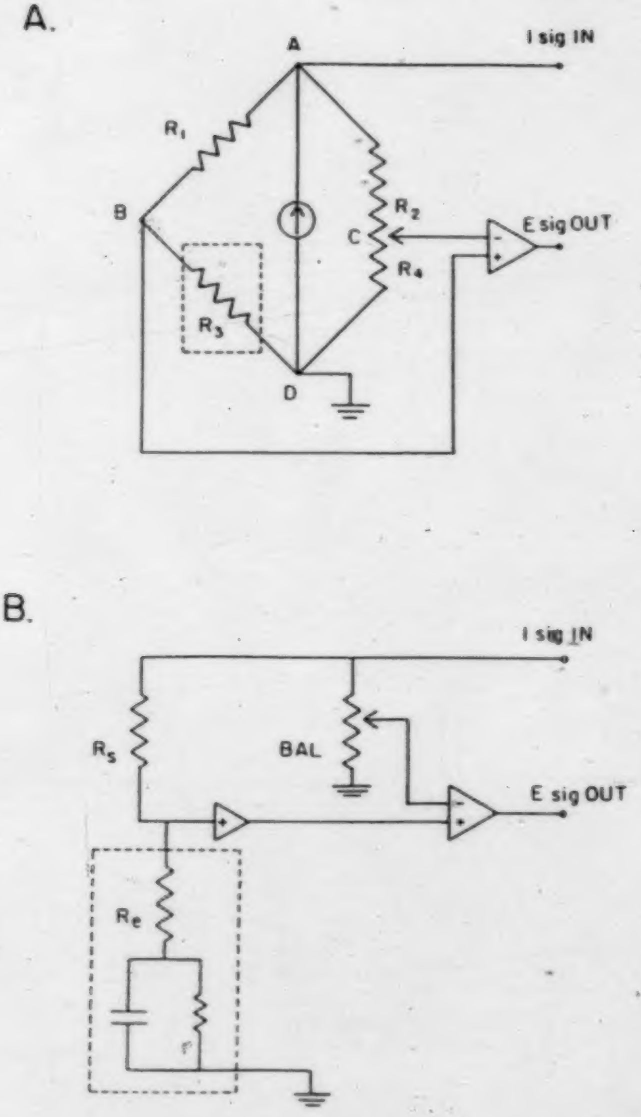

A.
A
I sig IN
R₁
B
R₂
C
E sig OUT
R₄
R₃
D
B.
I sig IN
Rs
BAL
E sig OUT
Re

Fig. 13. Bridge circuit for simultaneous recording and current injection drawn so as to: A. resemble the circuit devised by Wheatstone for measurements of resistance and, B. to show its likeness to more modern circuits (Purves, 1981). R_3 in A and the elements enclosed in the broken lined box in B correspond to the ganglion cell membrane and the recording electrode.

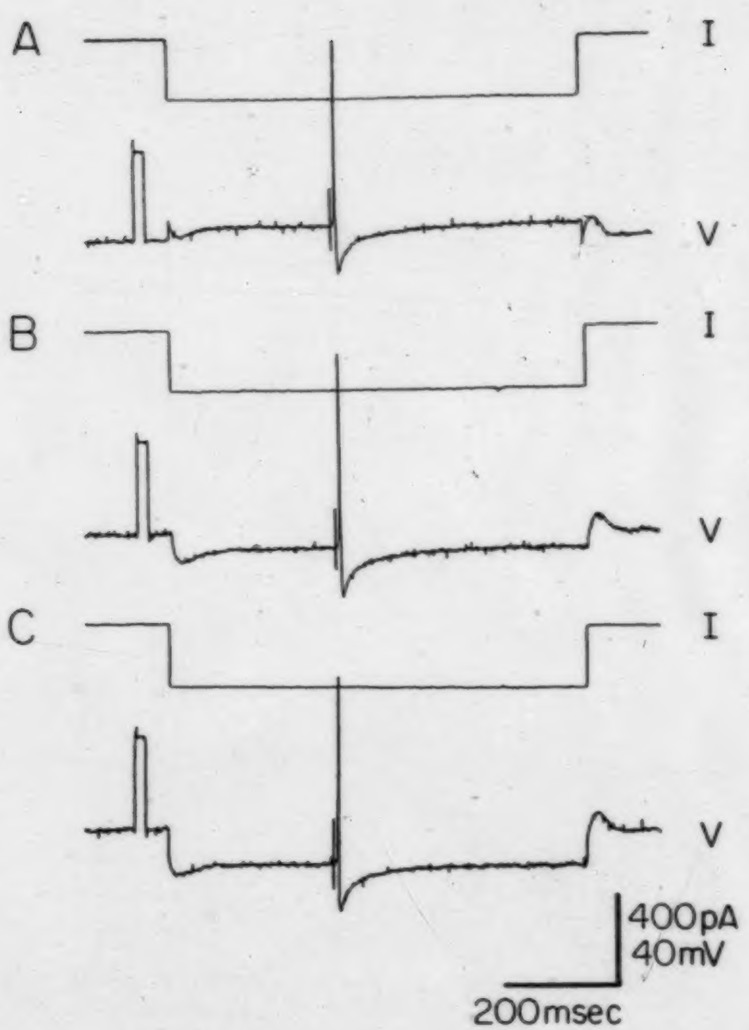


Fig. 14. Voltage response of a Rana catesbeiana sympathetic ganglion cell to a hyperpolarizing square-wave current pulse when the bridge was out of balance (not enough added resistance), A. in balance, B. and out of balance (too much added resistance), C. voltage calibration preceding the voltage response; 50 mV, 20 ms.

individual cells could not be identified. While the preparation was being approached, hyperpolarizing current pulses (approximately 0.01 nA, 800 msec) were injected through the balanced electrode every four seconds. Contact between cell membrane and electrode tip was indicated by a voltage offset in response to current on the Tektronix 5440 Oscilloscope. At this point, the Inchworm was advanced even more slowly while watching the Nicolet 2090 Digital Oscilloscope screen for a graded increase in the amplitude of the voltage deflection. The graded increase in the voltage offset was due to increasing resistance to current passage as the electrode tip pressed against the outer surface of the cell membrane. When a substantial amount of pressure was being applied, the tip of the electrode was oscillated by briefly (less than 1 sec) and abruptly turning up the capacitance compensation at the probe input. "Ringing" the electrode in this way vibrated the tip through the cell membrane. Once inside the cell it was often necessary to move the electrode forward or backward a few μms until a good seal, indicated by a steady resting potential, was obtained.

c) Data display and storage

The amplified signals were recorded on either the Nicolet 2090 Digital Oscilloscope or a Gould 2400S chart recorder as indicated in Fig. 12. Fast responses such as action potentials and e.p.s.p.'s were stored on 5.25 inch "floppy" computer discs by the Nicolet 2090. The data for the current-voltage curves (see Section C4) were also stored in this manner. Either Xidex Precision Flexible Discs (double sided, double density, soft sector) or similar discs by Dysan Corporation were used. Data stored in this way could be retrieved at a later date to be photographed or plotted using a Hewlett Packard 7015B X-Y Recorder.

The Gould 2400S was used to record slow drug responses. Three channels displayed a current trace and a high and low gain voltage trace. The low gain voltage record was used to determine the resting membrane potential of the cell while the current trace provided a record of any currents passed through the recording electrode. The frequency response of all three channels were adjusted from D.C. to 5 Hz.

4) Current-voltage curves

Current-voltage (I-V) curves were constructed for a large number of cells in order to determine their input resistances. Typically, eight square-wave hyperpolarizing current pulses (approx. 800 msec) of increasing strength were passed through a balanced (see Fig. 14B) recording electrode and the resulting voltage changes were recorded on the Nicolet 2090 (Fig. 15A). Each current value (nA) and the plateau amplitude of the corresonding voltage response (mV) were plotted as shown in Fig. 15B. The slope ($\Delta E/\Delta I$) of the linear portion of the curve was taken as the input resistance of the cell.

5) Addition of drugs

A similar superfusion system was used in these experiments as was described for the sucrose gap experiments (see Section 2B4). One addi-

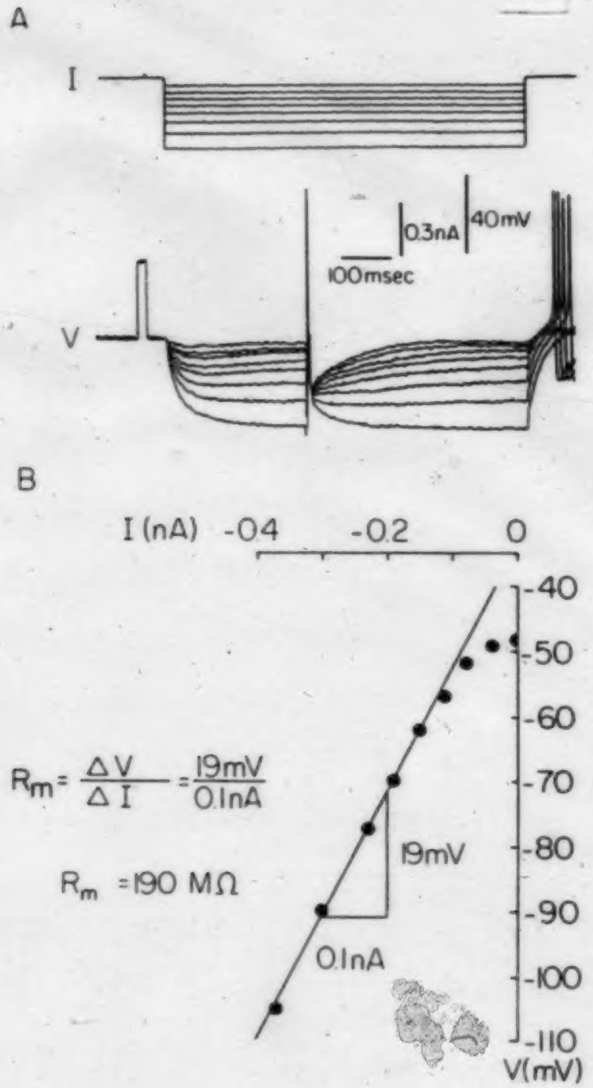


Fig. 15. Example of intracellular technique used to determine the input resistance (R_m) of a *Rana catesbeiana* sympathetic ganglion cell. A. series of eight square-wave hyperpolarizing current pulses and corresponding voltage responses in a B cell with bridge properly balanced. B. Current-voltage (I-V) plot of data shown in A. R_m was determined from the slope of the straight line portion of the I-V curve.

tional feature of the intracellular system was the interpositioning of an electrically switched solenoid valve (General Valve Corp., Cat. No. 1-47-900) downstream of the 3-way Luer valves but upstream of the needle valve tubing clamp (which regulated the flow of Ringer's solution into the intracellular bath at 0.1 - 0.5 ml. min^{-1}). The solenoid valve was positioned as close to the bath as was physically possible to enable very rapid and discrete drug additions. Also, since the valve was operated electrically instead of manually, this minimized the danger of dislodging the electrode from the impaled cell during solution changes. All other aspects of the intracellular superfusion system were identical to those of the sucrose gap system described in Section 2B4.

D. Solutions for electrophysiological studies

The composition of the normal frog ganglion Ringer's solution used for both the sucrose gap and intracellular experiments is indicated in Table 1. Deionized water (columns by Barnstead/Sybron Corp.) was used to prepare all aqueous solutions. The suppliers of the drugs and chemicals used for the electrophysiological studies are listed in the Appendix. Stock solutions of NaCl, KCl, $CaCl_2$ and TRIS (made with distilled water at 10X's final Ringer's solution concentration) were stored for up to three weeks in the cold room at 4°C. To make up the Ringer's solutions required for each experiment, appropriate amounts of these stock solutions were combined with anhydrous D-glucose and adjusted to pH 7.2. All drug solutions were also prepared fresh each day and whenever the buffering capacity of the Ringer's solution was exceeded, the solution was brought back to pH 7.2 with the appropriate amount of NaOH or HCl.

Desmethylimipramine (DMI, 500 nM) was added to the Ringer's solution for the majority of the adrenaline experiments using both the sucrose gap and intracellular techniques.

In many of the early sucrose gap experiments, and particularly in all the ones involving sodium orthovanadate (Na_3VO_4), sodium bisulphite (0.1 μM $NaHSO_3$/1 μM adrenaline) was used to retard oxidation of the adrenaline solutions. Although $NaHSO_3$ was effective in preventing the appearance of adenochrome for many hours and alone $NaHSO_3$ did not affect the resting membrane potential, its use was discontinued in later experiments. It was felt that the amount of adrenaline which oxidized in the dimly lit Faraday Cage had a negligible effect on the overall concentration of adrenaline in the bottle.

Another special provision made for the Na_3VO_4 experiments was that all Ringer's solutions were adjusted to pH 7.6 (from 7.2) because this was the extent to which 1 mM Na_3VO_4 shifted the pH of the TRIS buffered Ringer's solution. Any attempts to bring the pH of the 1 mM Na_3VO_4 Ringer's solution back down to 7.2 with acid (2 M HCl) resulted in the solution immediately turning a bright yellow color due to the formation of polymerized vanadate species (particularly decavanadate;

Macara, 1980).

The nicotinic antagonist d-tubocurarine (70 μM) was added to the Ringer's solutions for the slow i.p.s.p. experiments. This measure was necessary in order to block the postsynaptic generation of C spikes, the afterhyperpolarizations of which would obscure the true amplitude of the slow i.p.s.p.

All drugs and chemicals were completely soluble in normal Ringer's solution at the applied concentrations with the exception of 60 μM concentrations were made up in Ringer's solution by dissolving 2 mg of powder in 2 ml of DMSO and bringing the volume up to 100 ml with Ringer's solution. To control for any effects of the added DMSO, it was necessary to include 2% DMSO in all other drug solutions used in these experiments, including the control Ringer's solution. It was observed, however, that the ganglion preparation remained viable for many hours exposed to this amount of DMSO.

Due to the labile nature of the bee venom apamin, fresh solutions were made up immediately prior to performing the experiments. 1 mg of the peptide (powdered preparation, Sigma) was dissolved in 493 μl of distilled water to make a stock solution of 1 mM. The apamin was qualitatively bioassayed for activity on rabbit jejunum according to the method of Muller and Baer (1980). A 500 nM concentration of the active compound was then made up in normal Ringer's solution for the sucrose-gap experiments and in Ringer's solution containing 5 mM caffeine for the intracellular experiments. On a few occasions, the bioassay, the sucrose gap and the intracellular experiments were all performed on the same day. Apamin solutions were never stored and re-used.

E. NaK-ATPase experiments

1) Preparation of microsomal membranes containing NaK-ATPase activity

a) Solutions

The constituents of the Homogenizing, Wash and Suspending Buffers are indicated in Table 2. Deionized water (Barnstead/Sybron Crop.) was used for all biochemical solutions. All solutions were filtered through a Millipore filter with a pore size of 0.45 μM. The suppliers of the reagents used are specified in the Appendix.

b) Bovine brain

Whole bovine brain was obtained fresh from the abbatoir (Ouellette Packers Ltd., Edmonton) and transported in a container filled with Homogenizing Buffer and packed in crushed ice. The tissue was cut into small pieces and homogenized in 15X's volume of Homogenizing Buffer. This procedure was performed at 4°C using 2 x 10 sec. pulses of a Polytron Homogenizer (Brinkmann Instruments) fitted with a PT-20 generator and operated at setting 6.

A flow diagram of the centrifugation steps for the preparation of the microsomal membranes containing NaK-ATPase activity (NaK-ATPase preparation) is shown in Fig. 16. A refrigerated (at 4°C) Sorval RC2-B centrifuge equipped with a SS34 rotor was used to perform the

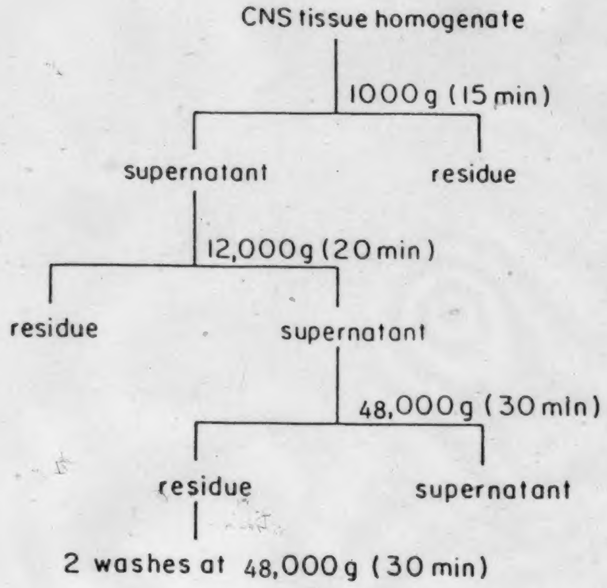

CNS tissue homogenate
1000 g (15 min)
supernatant
residue
12,000 g (20 min)
residue
supernatant
48,000 g (30 min)
residue
supernatant
2 washes at 48,000 g (30 min)

Fig. 16. Flow diagram for isolation of microsomal membranes from bovine brain and bullfrog CNS tissue containing NaK-ATPase activity.

following separations. Cellular debris was removed by centrifugation at 1,000Xg (3,000 rpm) for 15 min. The remaining supernatant was then centrifuged at 12,000Xg (10,000 rpm) for 20 min. to eliminate possible contamination from mitochondrial particles. This supernatant was centrifuged at 48,000Xg (20,000 rpm) for 30 min. centrifugations at this setting. The washed pellets were resuspended in Suspending Solution and stored in 1.0 ml aliquots at -20°C.

c) Frog CNS

Following decapitation, the brain and spinal cord of 12 bullfrogs were quickly removed and placed in pre-weighed vials in the cold room (4°C). The 1.734 grams of tissue obtained was then treated in a manner identical to that described in the preceding section for the bovine brain.

d) Frog sympathetic ganglia

Following decapitation (of the same frogs as in the preceding section), the paravertebral sympathetic ganglia (VIIIth - Xth) and interconnecting sympathetic chain were removed and placed in chilled pre-weighed vials in the cold room. The 88.3 milligrams of tissue obtained was homogenized in 20 volumes of ice-cold Suspending Solution using a teflon-glass tissue homogenizer. The resulting slurry was then sonicated to further dissociate the tissue. The homogenate was separated into 220 μl aliquots and frozen at -20°C. Due to the extremely small size of the sympathetic ganglia, it was physically impossible to perform any of the purification steps that were done with the bovine brain and frog CNS enzyme preparations.

2) Continuous enzyme linked spectrophotometric assay

Total ATPase actiivty was measured using a coupled optical assay similar to that previously described (Schoner et al. 1967; Charnock et al. 1977) at 340 nm. Fig. 17 schematically illustrates the reactions which link the hydrolysis of ATP to the oxidation of NADH. The composition of the Reaction Mixture is outlined in Table 3.

A Gilford 2400 spectrophotometer fitted with a jacketted ethylene glycol/water constant temperature bath was used in these experiments. The temperature of the spectrophotometric cell was controlled to 37 ± 0.2°C for the bovine brain experiments whereas assays of enzyme activity in frog CNS and sympathetic ganglion membranes were not temperature controlled and run at room temperature (20 - 24°C). After the Reaction Mixture and enzyme preparation had equilibrated for several minutes in the 3 ml plastic cuvette, the reaction was initiated by adding ATP to a final concentration of 1 mM. The ATP was thoroughly mixed with the cuvette contents with a small glass stirring stick. Equilibrium activity of the enzyme was assumed when oxidation of NADH became linear. Ouabain-sensitive NaK-ATPase activity was taken as the difference between total activity and that remaining in the presence of 6 mM ouabain.

3) Determination of Protein Content and Specific Activity of NaK-ATPase

The method of Lowry et al. (1951) was used to determine the

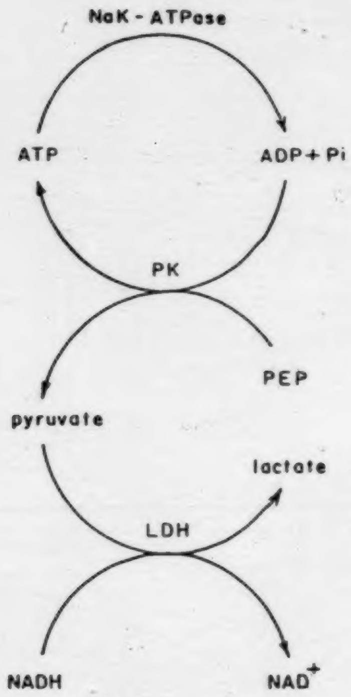

NaK - ATPase
ATP
ADP + Pi
PK
PEP
pyruvate
lactate
LDH
NADH
NAD$^+$

Fig. 17. Enzyme-linked reactions of spectrophotometric assay used to measure rate of ATP hydrolysis.

ATP - adenosine triphosphate

ADP - adenosine diphosphate

Pi - inorganic phosphate

PEP - phospho(enol)pyruvate

PK - pyruvate kinase

LDH - lactate dehydrogenase

NADH - β-nicotinamide adenine dinucleotide, reduced form

NAD$^+$ - β-nicotinamide adenine dinucleotide, oxidized form protein content in the bovine brain and frog CNS preparations. A standard curve of 5 points was constructed for each protein assay using bovine serum albumin. A Gilford Micro Sample 300 N spectrophotometer was used to measure the absorbance of the samples at 650 nm. From the standard curve, the precise amount of protein (enzyme preparation) added to each cuvette could then be established. This information was then used to calculate the specific activities of the enyzmes in the following way. The μmoles Pi produced per hour was determined knowing that a stoichiometric relationship of 1:1 existed between the amount of Pi produced by ATP hydrolysis to the amount of NADH oxidized to NAD^+. Use of the Beer-Lambert equation expressed below completed the calculation of the specific activities (μmoles Pi/mg protein/hr) of the frog CNS and bovine brain enzymes.

$$\Delta O.D. = \varepsilon . \Delta c . l$$

where: O.D. = optical density (or absorbance)

c = mM NADH

l = 1 cm light path (width of cuvette)

$\varepsilon = 6220\ c^{-1}.l^{-1}$

(absorption extinction co-efficient for NADH)

F. Data Analysis and statistics

All values presented in the Results for the electrophysiological studies have been expressed as a mean (X) ± standard error (S.E.) with the number of observations (n) indicated in parentheses. Unless otherwise specified, n represents the number of sympathetic ganglion preparations in the sucrose gap experiments and the number of cells in the intracellular experiments. For the biochemical studies, n denotes the number of times a particular measurement was made using one membrane preparation. Where appropriate, a paired or unpaired "student" t-test was employed to discern whether or not a statistically significant difference existed between two population means.

Unless clearly stated otherwise, all data was "normalized" for each experiment (preparation or cell). In this way, values have been presented as percentages (i.e. of a control response or a maximum response if a concentration-effect relationship was established). In other words, each experiment served as its own control. Under no circumstances were absolute values (e.g.'s mV, μmole Pi/mg protein/hr, etc.) compared between experiments.

Concentration-effect curves are illustrated with straight lines drawn from point to point. No effort was made to draw a smooth curve by eye through the points or to fit the data to a sigmoid function. However, for the purposes of determining the EC_{50} and some IC_{50} values, it was assumed that the points between 20-80% of maximum approximately followed a straight line and linear regression analysis was performed on the data in this range. The EC_{50} or IC_{50} values for each individual experiment were then used to calculate a geometric mean with 95% confidence limits. Where a straight-line relationship could not reasonably be assumed, linear regression analysis was not used and values of half-maximal effect were obtained directly from the graph. In these instances, a broken line is included in the figure to illustrate how the value was obtained.

CHAPTER III

RESULTS

A. Post synaptic effects of adrenaline in frog sympathetic ganglia

1. The adrenaline induced hyperpolarization recorded by the sucrose-gap technique

a) General

A number of investigators have used the sucrose-gap technique to study the adrenaline induced hyperpolarization (Ad_H) of bullfrog (*Rana catesbeiana*) sympathetic ganglia cells (Libet and Kobayashi, 1974; Koketsu and Nakamura, 1976; Weight and Smith, 1980; Rafuse and Smith, 1982). An adrenaline induced depolarization (Ad_D) has also been reported in these neurones, but it occurs less frequently than does the Ad_H (Koketsu and Nakamura, 1976; Rafuse and Smith, 1982). In the sucrose-gap experiments described in these chapters using leopard frog (*Rana pipiens*) sympathetic ganglia, only the Ad_H was observed. Ganglion preparations from the smaller frog species (*Rana pipiens*) were used in these sucrose-gap studies because it was found that the Ad_H in this tissue was less susceptible to desensitization than the Ad_H recorded from the bullfrog ganglia (Smith, 1984). Examples of Ad_H's and Ad_D's recorded from bullfrog sympathetic ganglia and Ad_H's from leopard frog ganglia are illustrated in Fig. 18. The Ad_H elicited by a 30 sec superfusion of adrenaline was observed to be a concentration-dependent effect. A maximum response amplitude (ranging from 500 μV to 3 mV depending on the preparation) was produced by 100 μM Ad. Usually, no response at all could be detected with 100 nM adrenaline. The log concentration-effect curve

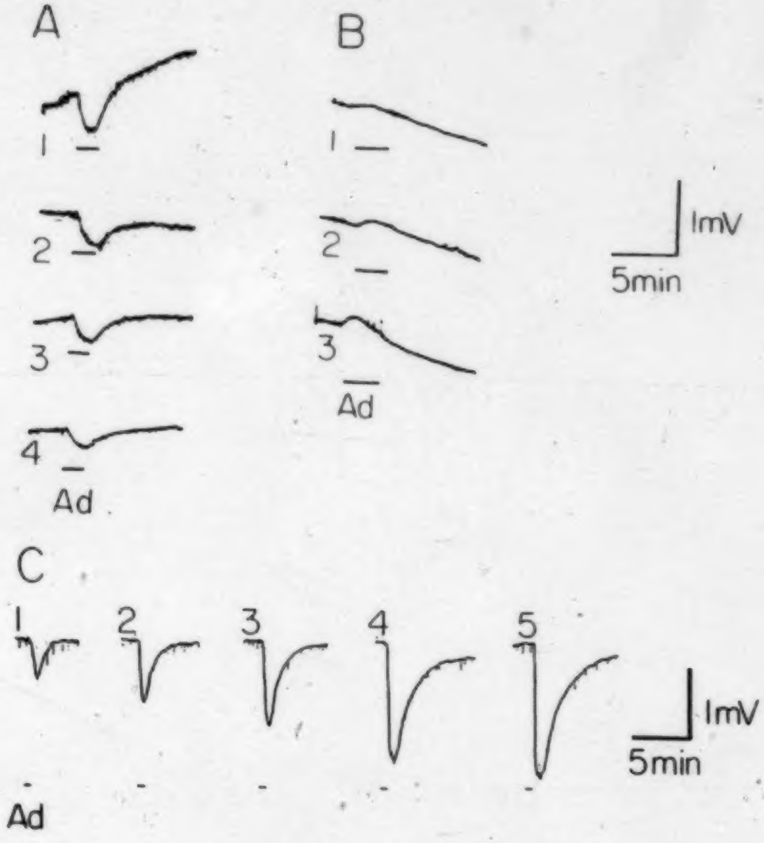


Fig. 18. Membrane potential responses to adrenaline recorded by the sucrose-gap technique. A. 1-4. Consecutive hyperpolarizing responses to 10 µM adrenaline in a *Rana catesbeiana* sympathetic ganglion. Note the apparent desensitization of the responses. B. Concentration-dependent depolarizations to adrenaline; B1. 3 µM, B2. 30 µM, B3. 300 µM in a *Rana catesbeiana* sympathetic ganglion. C. Concentration-dependent hyperpolarizations to adrenaline; C1. 200 nM, C2. 500 nM, C3. 2 µM, C4. 10 µM, C5. 20 µM in a *Rana pipiens* sympathetic ganglion. Upper calibration bars apply to columns A and B; lower calibration bars apply to row C. Black bars under traces indicate period of superfusion of adrenaline. Traces illustrated in row C were obtained in the presence of 500 nM DMI. All traces were obtained from a rectilinear pen recorder.

for adrenaline is illustrated in Fig. 19. The data was normalized to accommodate for the variability in size of the maximum Ad_H in each preparation. The EC_{50} for adrenaline, which was determined from the straight line established by linear regression analysis (between 20 and 80% of maximum response; see Materials and Methods), was 1.65 µM with 95% confidence limits of 1.23-2.21 µM (10 determinations on 5 preparations).

b) Effect of desmethylimipramine

In cat superior cervical ganglia, catecholamines remain largely unmetabolized (Adler-Graschinsky et al. 1984). In rat ganglia, ^{3}H-noradrenaline uptake has been reported to be inhibited by the catecholamine uptake blocker, desmethylimipramine (DMI; IC_{50} approx. 10 nM; Hanbauer et al. 1972). In an effort to investigate if such a mechanism for catecholamine inactivation exists in frog ganglia, the effect of DMI was examined on the Ad_H. It was typically observed that 500 nM DMI potentiated a submaximal Ad_H (elicited by 1 µM Ad) by over 100%. The log concentration-effect curve for adrenaline in the presence of 500 nM DMI is illustrated in Fig. 19. The EC_{50} for adrenaline (in the presence of DMI) was calculated to be 270 (220-340) nM (8 determinations on 5 preparations). Use of the unpaired t-test demonstrated that this EC_{50} was statistically different than the EC_{50} for adrenaline in the absence of DMI ($p<0.001$). DMI (500 nM) was not observed to have any intrinsic action of its own and it had no effect on the amplitude of the maximal Ad_H. Thus, unless otherwise

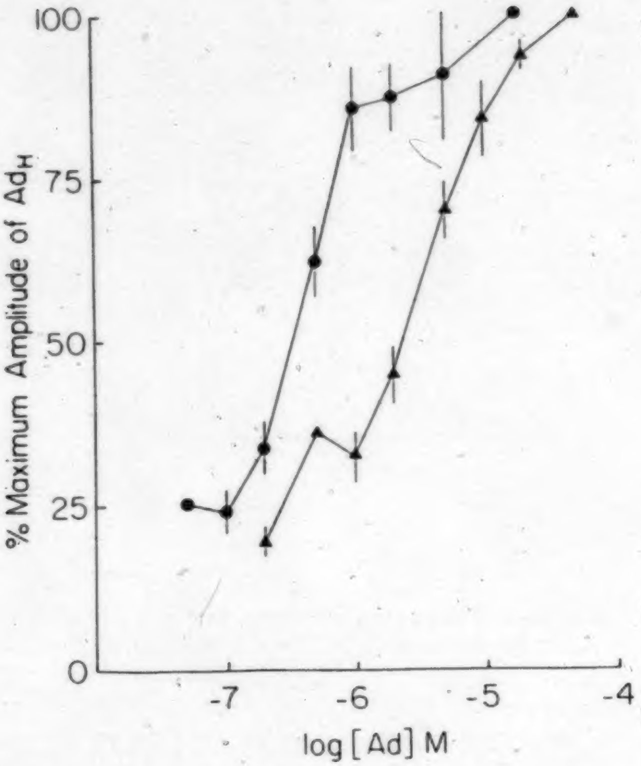


Fig. 19. Log concentration-effect curve for adrenaline induced hyperpolarization (Ad_H) of Rana pipiens sympathetic ganglia recorded by means of the sucrose-gap technique. Circles: Ad_H in presence of 500 nM DMI (n=8), triangles: Ad_H in absence of DMI (n=10). Each point indicates the normalized mean with standard error bars.

stipulated, this concentration of DMI was routinely used to potentiate the Ad_H in all sucrose-gap experiments. Also, 1 μM adrenaline (in the presence of 500 nM DMI) was used as a test concentration of adrenaline in many experiments because it produced a large but submaximal Ad_H.

2. Intracellular studies with adrenaline

In spite of the relative ease in obtaining Ad_H's in ganglia of both frog species using the sucrose-gap technique, convincing Ad_H's were not satisfactorily recorded from Rana catesbeiana using conventional intracellular techniques. This corroborates the findings of other investigators working with frog sympathetic ganglia (Koketsu and Nakamura, 1976; Kuba and Koketsu, 1978). A number of possible reasons for this negative result are reviewed in the Discussion.

Stable intracellular recordings could be obtained from B and C cell soma for several hours. In order to test the effects of adrenaline, it was necessary to hold healthy cells for at least 15 min. Although individual cells could not be visualized, most recordings were obtained from cells of the superficial layers so that diffusion of superfused drugs to the impaled cell would not be seriously impeded. As mentioned in the Materials and Methods, B and C neurones were identified according to the conduction velocities ($m.sec^{-1}$) of antidromically evoked potentials. Representative examples of B and C spikes are shown in Fig. 20 A and C. Stimulation of the rostral portion of the sympathetic chain (see Materials and

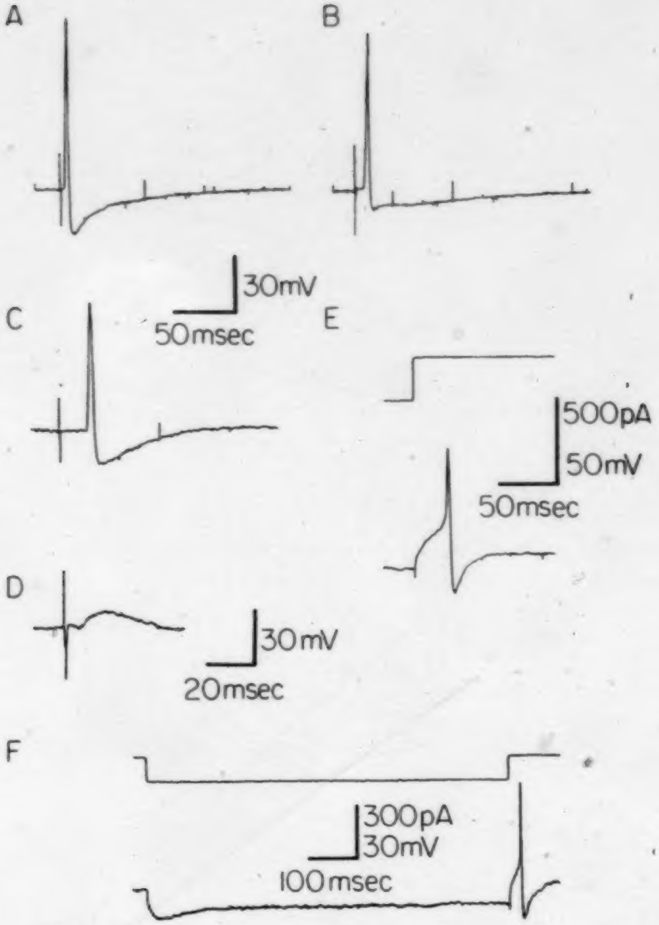


Fig. 20. Intracellular recordings from B and C cells of Rana catesbeiana sympathetic ganglia. A. antidromically evoked B spike; B. orthodromically evoked B spike; C. antidromically evoked C spike; D. orthodromically evoked fast e.p.s.p. recorded from B cell; E. B spike evoked by direct injection of depolarizing current (rheobase); F. B spike evoked following the termination of a hyperpolarizing current pulse (anodal break spike). Top-left calibration bars for traces A, B and C. Records A and B were taken from the same cell. Records C, D, E and F were taken from four different cells. DMI (500 nM) was present in all Ringer's solutions.

Methods) evoked either an e.p.s.p. which usually reached threshold for the production of an orthodromic action potential. A subthreshold fast e.p.s.p. is illustrated in Fig. 20D. Note from the example given in Fig. 20B that the AP_{AH} of the orthodromic spike was obscured by the underlying fast e.p.s.p. Normally the amplitude of the orthodromic spike was less than the amplitude of the antidromic spike in the same cell due to the shunting effect of the fast e.p.s.p. (Blackman, et al., 1963). Action potentials could also be directly evoked by injecting depolarizing current pulses through the recording electrode. Another method was to produce "anodal break" action potentials by injecting hyperpolarizing current. Examples are depicted in Fig. 20E and F.

Only B and C cells with antidromically evoked action potential amplitudes greater than 70 mV and steady resting membrane potentials greater than -40 mV were used for testing the effects of adrenaline. Table 4 summarizes the essential properties of the B and C cells from which stable intracellular recordings were obtained. Prospects for detecting somal membrane potential changes in response to adrenaline were considered to be further improved if the cell input resistance was as large as possible (up to 500 MΩ). The electrode resistance was usually limited to less than 100 MΩ, since it was required that constant current pulses of variable amplitude be passed through the recording electrode in order to establish a current-voltage (I-V) relationship for each cell. High resistance

Table 4

Electrophysiological characteristics of B and C neurones of *Rana catesbeiana* sympathetic ganglia - intracellular

Cell Type	Cond.Vel. $(\mathrm{m.sec}^{-1})^{a}$	APampl. (mV)	AP ampl (mV)	AP dur (msec)	E_m (mV)	R_m [b] (MΩ)
B	1.85±0.16 n=57	80.1±1.9 n=54	26.5±0.7 n=54	192±15 n=54	50.3±0.8 n=54	194±28 n=22
C	0.24±0.03 n=14	79.9±1.2 n=8	25.7±1.3 n=8	125±23 n=8	51.3±3.2 n=8	167±33 n=2

[a,b] For calculations of antidromically evoked action potential conduction velocity (Cond.Vel.) and input resistance (R_m) see Materials and Methods.

electrodes of greater than 100 MΩ generally exhibited poor current passing properties and consequently could not be used in these experiments. The slow flow rate of the superfusing Ringer's solutions (0.1-0.5 ml. min^{-1}; see Materials and Methods) necessitated an estimation of the diffusion time for the adrenaline to reach the impaled cell. This was determined to be in the range of 30 sec to 3 min depending on the flow rate. I-V curves were constructed before and during the estimated period when the adrenaline was in contact with the impaled cell, in order to examine whether any change in input resistance (R_m) occurred during exposure to the adrenaline. The method for constructing the I-V curves is described in the Materials and Methods.

Adrenaline (2 to 100 μM) was superfused for 15 to 90 sec over ganglia in which the recording conditions and properties of the 54 B and 8 C cells studied were deemed to be optimal. This concentration range of adrenaline failed to produce any reproducible changes (depolarizations or hyperpolarizations) in the resting potential of any of these cells. Furthermore, no change in R_m was detected during the adrenaline application. The I-V curves before and during the adrenaline superfusion were generally superimposeable. Representative examples of I-V curves for B and C cells are given in Fig. 21. It would appear from these attempts that an Ad_H is extremely difficult (or impossible) to record from a single cell soma of the bullfrog sympathetic ganglia using conventional intracellular techniques.

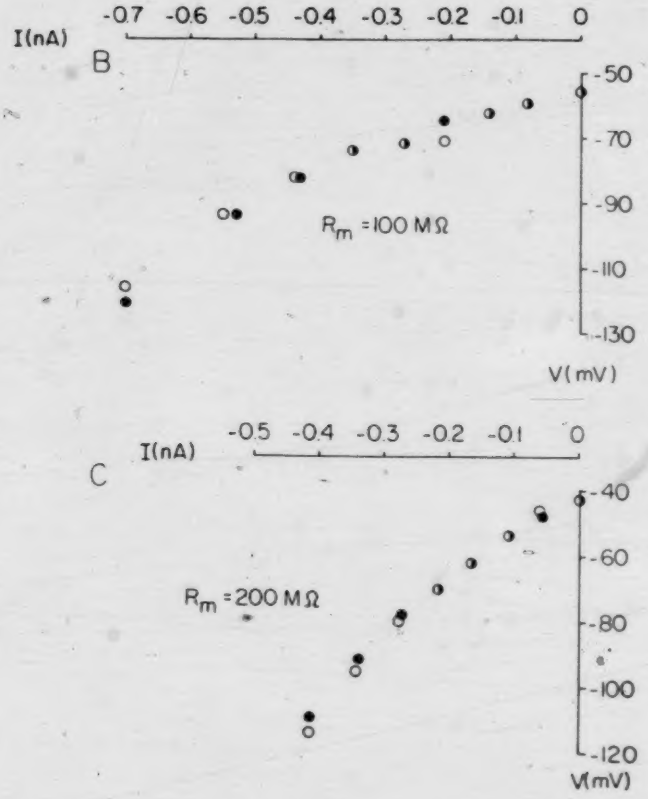


Fig. 21. Current-voltage (I-V) curves in the presence and absence of adrenaline obtained by intracellular recordings from a representative B and C cell of Rana catesbeiana sympathetic ganglia. Upper figure shows the I-V curve obtained from a B cell with resting membrane potential (E_m) of -56 mV and input resistance of (R_m) of 100 MΩ. Neither the E_m nor the R_m were changed by 10 μM Ad superfused for 90 sec. Lower figure shows the I-V curve obtained from a C cell with E_m of -42 mV and R_m of 200 MΩ. Neither the E_m nor the R_m were changed by 50 μM Ad superfused for 15 seconds. In both figures closed circles indicate control responses while open circles indicate responses in the presence of Ad. DMI (500 nM) was present in all Ringer's solutions.

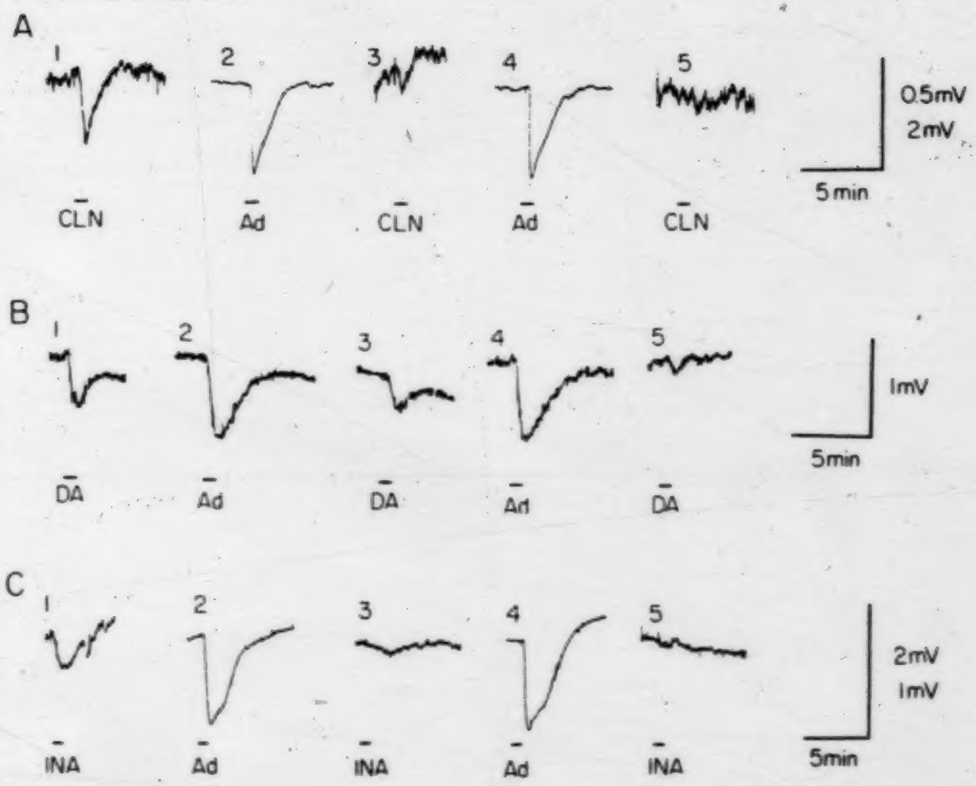
B. Identification of the adrenoceptor which mediates the adrenaline induced hyperpolarization

In the post-synaptic neurones of mammalian sympathetic ganglia, α- and β-adrenoceptor mediated events have been described (DeGroat and Volle, 1966; Brown and Caulfield, 1979; Brown and Dunn, 1983). Dopamine receptor mediated actions have also been reported (Dun et al. 1977). The noradrenaline induced hyperpolarization observed in rat superior cervical ganglia is proposed to be mediated by an α_2-adrenoceptor (Brown and Caulfield, 1979). Much less is known about adrenoceptors in amphibian ganglia. Koketsu and Nakamura (1976) have reported that the adrenaline induced hyperpolarization (Ad_H) in Rana catesbeiana sympathetic ganglia is selectively antagonized by phenoxybenzamine (15 μM). The purpose of the following study was to elucidate the adrenoceptor responsible for the mediation of the Ad_H recorded by the sucrose-gap technique in Rana pipiens sympathetic ganglia.

1. Adrenoceptor agonist studies

In addition to adrenaline, several adrenergic agonists were observed to produce hyperpolarizing responses in Rana pipiens sympathetic ganglia. Hyperpolarizing responses were obtained to dopamine (1 μM), isoprenaline (1-100 μM), α-methylnoradrenaline (10 nM - 10 μM) and very occasionally to clonidine (100 nM - 10 μM). Representative responses to these agonists are shown in Fig. 22. No responses could be elicited by the application of methoxamine (1 μM - 1 mM).

Fig. 22. Desensitization of clonidine (CLN), dopamine (DA) and isoprenaline (INA) responses recorded from Rana pipiens sympathetic ganglia by means of the sucrose-gap method. A1. Initial response to 100 nM CLN; A2. Initial response to 1 μM adrenaline (Ad); A3. Second response to CLN was only 40% of initial response; A4. Second response to Ad was same as initial Ad_H; A5. Third application of 100 nM CLN produced no response at all. Responses A1-5 were evoked with a 15 min interval between drug applications. 0.5 mV calibration refers to CLN, 2 mV calibration refers to Ad_H. B1. Initial response to 1 μM DA; B2. Initial response to 1 μM Ad; B3. Second response to DA was reduced to 64% of control; B4. Second response to Ad was unchanged from control; B5. Third response to DA was reduced to 33% of control. Responses B1-5 were evoked with a 15 min interval between drug applications. C1. Initial response to 1 μM INA; C2. Initial response to 1 μM Ad; C3. Second response to INA was only about 30% of control response; C4. Second response to Ad was same as control amplitude; C5. Third application of 1μM INA produced no response. Responses were evoked with a 20 min interval between drug applications. 2mV calibration refers to Ad_H, 1 mV calibration refers to INA. Traces from three different preparations were obtained from a rectilinear pen recorder. Black bars under traces indicate period of superfusion with Ad, DA, CLN or INA. Ringer's solution in each experiment contained 500 nM DMI.


A
1
2
3
4
5
0.5mV
2mV
5 min
CLN
Ad
CLN
Ad
CLN
B
1
2
3
4
5
1mV
5min
DA
Ad
DA
Ad
DA
C
1
2
3
4
5
2mV
1mV
5min
INA
Ad
INA
Ad
INA

The responses to dopamine (DA), isoprenaline (INA) and clonidine (CLN) were especially prone to a desensitization type of phenomenon. Repeated 30 sec superfusions of the agonists (even when they were spaced by 30 min) resulted in a gradual (e.g. DA) to very rapid (e.g.'s INA and CLN) decline in response amplitude. The hyperpolarizations obtained to CLN were particularly small such that a response measuring greater than 250 μV was observed in only one of six preparations (Fig. 22A). It should be noted that CLN failed to produce a response larger than 250 μV in 5 preparations regardless of whether 500 nM DMI was present or not (cf. Williams, et al., 1985). The marked tendancy of the CLN, DA and INA responses to diminish in amplitude to repeated drug applications made it impossible to determine a concentration-effect relationship for these agonists. Furthermore, in the majority of the experiments performed with antagonists it was not possible to obtain unambiguous results. The effectiveness, or lack of effectiveness, of the antagonist was obscured by the progressive decline in the control response amplitude that would have occurred even in the absence of the antagonist. It should also be noted that the gradual disappearance of the responses to CLN, DA and INA was an irreversible phenomenon. No measure of recovery was ever observed, regardless of the amount of time that the preparation was washed following loss of the response. The irreversible loss of these agonist responses was not due to general deterioration of the ganglion preparation since Ad_H's of constant amplitude could still be elicited.

In contrast to the other agonists tested, the α_2-agonist, α-methylnoradrenaline, produced a hyperpolarization (αMeNA$_H$) that was of similar amplitude and time course to the Ad$_H$ and that was no more prone to desensitization than was the Ad$_H$. A representative control response to 1 μM αMeNA is shown in Fig. 23A. This figure also shows that the α_2-adrenoceptor antagonist idazoxan (500 nM) reduced the amplitude of the αMeNA$_H$ (elicited with 1 μM αMeNA) to 11% of control (see also next section of Results). Analysis of the concentration-effect relationship for this agonist, in the presence of 500 nM DMI, revealed that the concentration of αMeNA which would produce a half-maximal response (EC$_{50}$) was 310 (130-730) nM (n=5). The log concentration-effect curve for αMeNA, in the presence of 500 nM DMI, is illustrated in Fig. 23B.

2. Cross-desensitization studies

It is commonly accepted among pharmacologists, that if a receptor mediated response to an agonist undergoes desensitization, then the response to another agonist acting on the same receptor will also be diminished (Gaddum, 1953; Rang, 1973; Moochhala and Sawynok, 1984). This phenomenum of cross-desensitization is often used as evidence to contend that two or more agonists act at the same receptor. Conversely, lack of cross-desensitization, where one agonist induced response is preserved while the other agonist induced response declines, would be taken to suggest that the agonists are acting

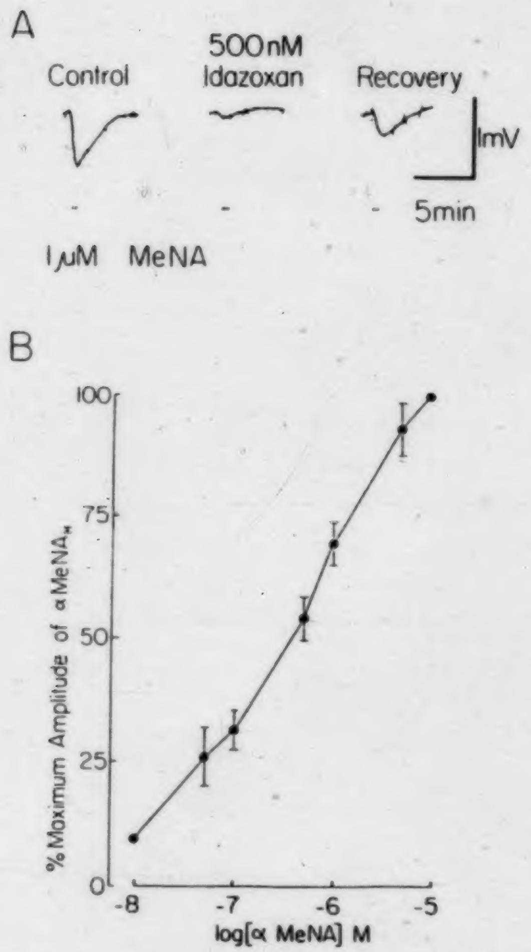


Fig. 23. **Effect of idazoxan and concentration-effect curve for** α**MeNA-induced hyperpolarization (**α**MeNA**$_H$**) from** ***Rana pipiens*** **sympathetic ganglia by means of the sucrose-gap technique. A. Control** α**MeNA**$_H$ **(to 1 μM** α**MeNA) response to** α**MeNA following 5 min exposure to 500 nM idazoxan; recovery** α**MeNA**$_H$ **following 65 min washout of idazoxan. Black bars under responses indicate period (30 sec) of** α**MeNA application. DMI (500 nM) was present in the Ringer's solutions for this experiment. Traces were obtained from a rectilinear pen recorder. B. Log concentration-effect curve of hyperpolarization to** α**MeNA in the presence of 500 nM DMI. Each point represents the normalized mean (with standard error bars) of 5 separate experiments. The EC**$_{50}$ **was determined by the linear regression method described in the** ***Materials*** **and** ***Methods*****.**

through diferent receptors or at least different "receptor sites".

It was observed in the course of this study that, for a given sucrose-gap preparation, the Ad_H usually remained at a constant amplitude when 1 μM adrenaline was superfused for 30 sec at a 30 min interval. However, if CLN, DA or INA were also applied at a 30 min interval (but 15 min out of phase with the adrenaline applications), it was consistently observed that while the Ad_H remained constant or declined very slowly, the CLN_H, DA_H or INA_H declined very rapidly. Selected experiments are illustrated in Figs. 22A, B and C.

3. Adrenoceptor antagonist studies

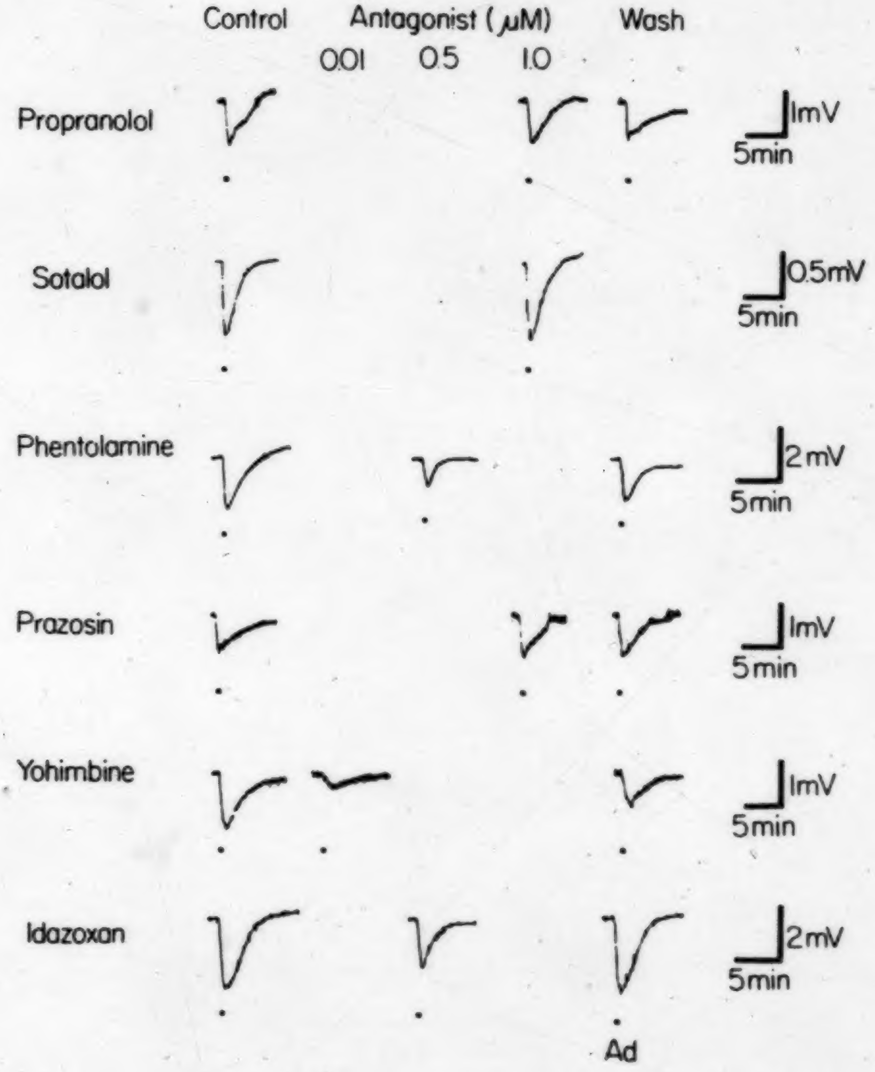
a) Ineffective antagonists

Various adrenoceptor antagonists were tested against the Ad_H (to 1 μM adrenaline in the presence of 500 nM DMI). Fig. 24 illustrates representative examples of the effects observed with each antagonist. Equimolar concentrations (1 μM) of the non-selective β-blockers propranolol (n=4) and sotalol (n=3) applied for at least 25 min produced no measureable effect on the amplitude or duration of the Ad_H. Likewise, 1 μM prazosin (α_1-blocker) was found to leave the Ad_H unchanged when applied to the ganglion for at least 40 min (n=3). The DA antagonist, chlorpromazine (1 μM), was observed to produce a slight (but statistically insignificant) reduction in the amplitude of the Ad_H to 92.2 + 5.3% of control after 30 min (n=4).

b) Alpha-2 adrenoceptor antagonists

All three α_2-antagonists tested produced variable but marked

Fig. 24. Effect of adrenoceptor antagonists on the Ad_H recorded from Rana pipiens sympathetic ganglia by means of the sucrose-gap technique. Left hand column; control response to 1 μM adrenaline recorded in the presence of 500 nM DMI. Centre columns; Ad_H responses recorded after various times in indicated concentrations of antagonists. From top to bottom: Ad_H after 25 min in 1 μM propranolol, Ad_H after 45 min in 1 μM sotalol, Ad_H after 25 min in 500 nM phentolamine, Ad_H after 35 min in 1 μM prazosin, Ad_H after 45 min in 10 nM yohimbine, Ad_H after 15 min in 500 nM idazoxan. Concentrations of antagonists are indicated at the top of centre columns. Right hand columns; Ad_H responses recorded after various periods of washout of antagonists with normal DMI Ringer's solution. From top to bottom: Ad_H after 10 min washout with propranolol, Ad_H after 20 min washout of phentolamine, Ad_H after 160 min washout of prazosin, Ad_H after 65 min washout of yohimbine, Ad_H after 30 min washout of idazoxan. Voltage and time calibrations at right refer to each experiment (separate preparations for each antagonist). Black bar under responses indicates period of superfusion of adrenaline. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in all Ringer's solutions.


Control
Antagonist (μM)
0.01
0.5
1.0
Wash
Propranolol
1mV
5min
Sotalol
0.5mV
5min
Phentolamine
2mV
5min
Prazosin
1mV
5min
Yohimbine
1mV
5min
Idazoxan
2mV
5min
Ad

inhibitions of the Ad_H. The amplitude of the Ad_H was reduced by the non-selective (α_1 and α_2) blocker phentolamine (IC_{50}=530 nM, n=4), and the α_2-selective blockers idazoxan (IC_{50}=590 nM, n=4) and yohimbine (IC_{50}=6.2 nM, n=4). The log concentration-effect curves from which these IC_{50}'s were determined are illustrated in Fig. 25. While the antagonism of the Ad_H by phentolamine and idazoxan was rapidly reversible, the blockade produced by even low concentrations of yohimbine required several hours of washing for full recovery to occur. It was consistently observed that when idazoxan was superfused over the ganglion, a transient depolarization resulted. The membrane potential always returned to baseline before adrenaline was applied. No changes in membrane potential were seen with yohimbine, phentolamine or any of the other antagonists used.

c) Dopamine and alpha-2 receptors?

It has been proposed that the DA_H in mammalian sympathetic ganglia is mediated via an α_2-adrenoceptor (Brown and Caulfield, 1979; Ashe and Libet, 1982) rather than by a DA receptor (Dun et al. 1977). Since the DA_H obtained in Rana pipiens sympathetic ganglion was especially prone to desensitization, the studies with antagonists were difficult to interpret. In 7 experiments, the DA_H (to 1 μM DA) was observed to gradually diminish in the presence of equimolar concentration of chlorpromazine. Since the amplitude of successive DA_H's declined with time, it was impossible to ascertain whether this effect was due to receptor antagonism or desensitization. In two experiments

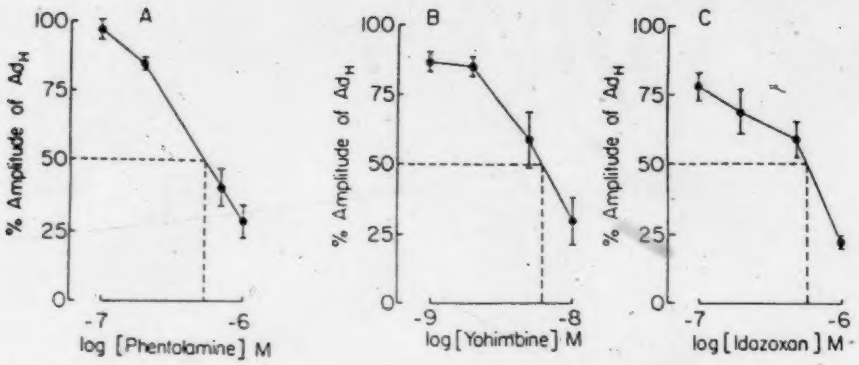


Fig. 25. Log concentration-effect curves for: A. phentolamine, B. yohimbine and C. idazoxan induced antagonism of the hyperpolarization induced by 1 μM adrenaline (Ad_H) in the presence of 500 nM DMI. Data was obained from Rana pipiens sympathetic ganglia using the sucrose-gap technique.

in which a reasonably consistent, albeit small, DA_H could be elicited, it was possible to demonstrate selective antagonism of the Ad_H by 10 nM yohimbine when the small DA_H was unaffected. Fig. 26 illustrates records of one of these experiments. While it is unclear whether the DA_H in these ganglia is sensitive to DA antagonists (i.e. chlorpromazine), it is unlikely that the DA_H is mediated by an α_2-adrenoceptor.

d) Beta mediated effects?

Depolarizations to noradrenaline and isoprenaline have previously been reported in mammalian sympathetic ganglia (DeGroat and Volle, 1966; Brown and Dunn, 1983) and these effects have been attributed to β-receptor activation. Sensitivity to antagonists was not examined with the infrequently recorded Ad_D observed in Rana catesbeiana ganglia (Koketsu and Nakamura, 1976; Rafuse and Smith, 1982). If a β-adrenergic depolarizing effect occurred concurrently with the Ad_H in the Rana pipiens ganglia, it might have been expected that the Ad_H would get larger in the presence of β-blockers. It has been shown in an earlier section of the Results that propranolol and sotalol (both at 1 μM) had no measureable effect on the Ad_H (to 1 μM adrenaline). Furthermore, no depolarizations could be produced by INA (100 nM - 1 mM) or by high concentrations of adrenaline (up to 50 μM) when the Ad_H was completely blocked by 100 nM or 1 μM yohimbine. The β_2-adrenergic depolarization in rat superior cervical ganglia described by Brown and Dunn (1983) was only observed in freshly

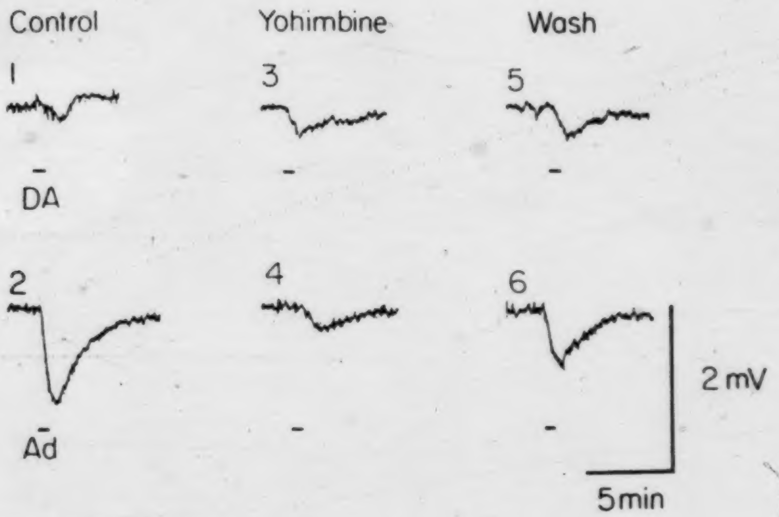


Fig. 26. Effect of yohimbine on DA_H and Ad_H from **Rana pipiens** sympathetic ganglia by means of the sucrose-gap technique. 1. Control response to 1 μM dopamine. 2. Control response to 1 μM adrenaline. 3. DA_H recorded after 25 min superfusion with 10 nM yohimbine. 4. Ad_H recorded after 45 min in yohimbine. 5. DA_H recorded 17 min after washout of 10 nM yohimbine. 6. Ad_H recorded 65 min after yohimbine washout. Black bars under traces indicate periods of superfusion with adrenaline (Ad) or dopamine (DA). Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in all Ringer's solutions.

dissected preparations. In all of the sucrose-gap experiments with Rana pipiens ganglia described in this thesis, only hyperpolarizing responses were observed regardless of whether the preparations were fresh or stored overnight at 4°C. It would therefore seem reasonable to suggest that β-adrenergic effects cannot be observed in Rana pipiens sympathetic ganglia under the experimental conditions of these sucrose-gap experiments.

C. Examination of the possible involvement of catecholaminergic transmission in slow synaptic inhibition in amphibian sympathetic ganglia

It has been proposed that the atropine-sensitive slow inhibitory post-synaptic potential (slow i.p.s.p.) recorded in Rana catesbeiana sympathetic ganglia results from a disynaptic mechanism involving the release of a catecholamine from an interneurone (Tosaka et al. 1968; Libet and Kobayashi, 1974; see Introduction and Fig. 3). Alternatively, it has been argued that ACh can act directly on muscarinic receptors on the primary post-synaptic neurone to produce the slow i.p.s.p. (Weight and Padjen, 1973b; Horn and Dodd, 1981). In order to examine which of these mechanisms underlies the slow i.p.s.p. in Rana pipiens, a number of pharmacological agents were employed to compare their effects on the hyperpolarizing responses to: i) the direct application of the adrenergic agonist adrenaline (Ad_H), ii) the direct application of the muscarinic agonist methacholine (MCh_H) and iii) preganglionic stimulation (slow i.p.s.p.).

1. The slow inhibitory postsynaptic potential and the methacholine induced hyperpolarization

A slow i.p.s.p. could be generated in *Rana pipiens* sympathetic neurones by stimulating the C fibres in the rostral portion of the VIIIth spinal nerve (Tosaka et al. 1968; Horn and Dodd, 1983; see Fig. 7 in *Materials and Methods*). Continuous superfusion of a Ringer's solution containing 70 μM d-tubocurarine (d-TC) was necessary to minimize the amplitude of the nicotinic fast excitatory post synaptic potential (e.p.s.p.) which would otherwise reach threshold for the generation of a postsynaptic action potential. Either a single shock or a train of repetitive stimuli were applied to the VIIIth spinal nerve to induce a slow i.p.s.p. A number of slow i.p.s.p.'s recorded by the sucrose-gap technique and evoked by a variety of stimulation parameters are illustrated in Fig. 27. A 1 sec train of 1 msec pulses at 10 Hz was found to provide an optimum response and these parameters were used for the majority of the following experiments. Since the slow i.p.s.p. recorded by the sucrose-gap technique is a population response, supramaximal voltage intensities were applied in order to recruit as many neurones as possible and thus the same number of neurones for each stimulation. Slow i.p.s.p.'s were never evoked more frequently than once per minute. Slow i.p.s.p.'s evoked by the optimum stimulation parameters were typically 0.5 to 3 mV in amplitude and 3 to 5 sec in overall duration.

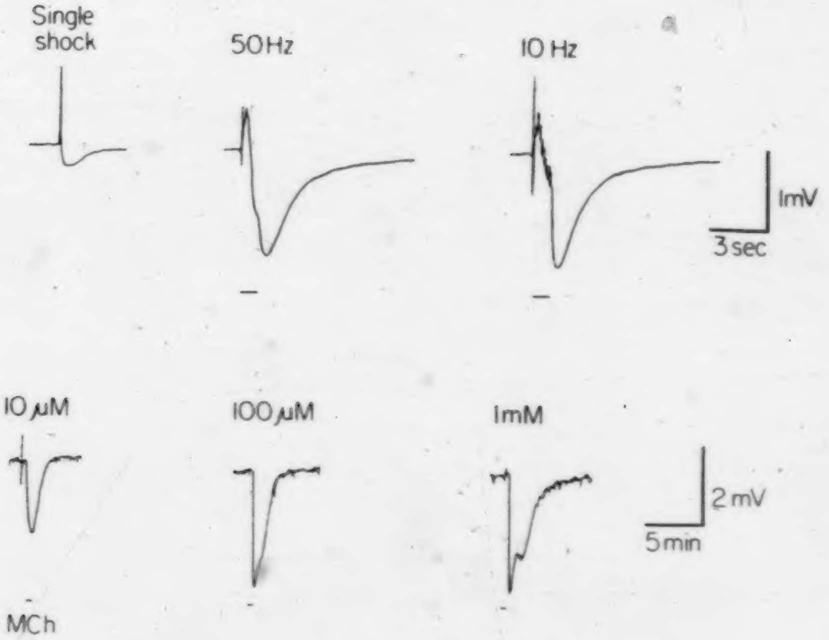


Fig. 27. Slow i.p.s.p.'s and MCh_H's recorded from two spearate *Rana pipiens* sympathetic ganglia by means of the sucrose-gap technique. Upper traces: slow i.p.s.p.'s elicited by; single shock; tetanic stimulation (1 msec pulses, 50 Hz for 1 sec); tetanic stimulation (1 msec pulses, 10 Hz for 1 sec). Black bars under slow i.p.s.p.'s indicate periods of stimulation. Lower traces: MCh_H's produced by 10 μM; 100 μM and 1 MM MCh. Black bars under MCh_H's indicate period (30 sec) of superfusion. Bandwidth was D.C. to 15 Hz for slow i.p.s.p.'s and D.C. to 5 Hz for MCh_H's. All traces were obtained from a rectilinear pen recorder and retouched for clearer presentation.

In _Rana pipiens_ the muscarinic response to methacholine (MCh, in the presence of 70 μM d-TC) was almost wholly a hyperpolarization (MCh_H). This is different to what is observed in _Rana catesbeiana_ where the response to superfused MCh involves a long depolarization preceeded by a transient hyperpolarization (Weight and Smith, 1980). Sometimes a small depolarization was detected at the peak of the MCh_H in _Rana pipiens_ (Fig. 27). This appeared as if a "bite" had been taken out of the MCh_H. Very often the MCh_H was also followed by a slower after-depolarization. In the absence of any anticholinesterase agents, the maximal MCh_H (to 1 mM MCh) was typically 1.0 to 4.0 mV in amplitude and 1.5 to 4 min in overall duration. The MCh_H evoked by a 30 sec application of 10 μM MCh was a submaximal response. Responses to 100 μM and 1 mM MCh were observed to be of maximal amplitude, but the MCh_H's elicited by 1 mM MCh were generally longer in duration than those elicited by 100 μM MCh. Sample responses to these concentrations of MCh are shown in Fig. 27.

2. _Comparison of the sensitivities of the Ad_H, MCh_H and slow i.p.s.p. to desmethylimipramine_

Desmethylimipramine (DMI) is widely accepted to block nerve terminal re-uptake of noradrenaline and adrenaline in a wide variety of neural systems (Iversen, 1975). DMI has also been shown to inhibit uptake of tritiated noradrenaline into superior cervical of rat in organ culture (IC_{50} approx. 10 nM, Hanbauer et al. 1972). It has been shown in the first section of the _Results_ that 500 nM DMI reduced the

EC_{50} for the Ad_H by a factor of 6 (from 1.65 to 0.27 μM). In the present experiments, 500 nM DMI was observed to potentiate the amplitude of the Ad_H (to 500 nM Ad) to 169.1 ± 4.0% of control after at least 35 min (n=3). The duration of the Ad_H was also increased to 155.6 ± 8.0% of control (n=3). A representative experiment is illustrated in Fig. 28. This potentiation of the Ad_H (amplitude and duration) was essentially irreversible since it was observed to persist for up to 2 hours following the washout of DMI.

According to the hypothesis of Libet and Kobayashi (1974; see Introduction), the MCh_H should result from the receptor-mediated (muscarinic) release of adrenaline from an interneurone. If this were the case, the MCh_H should be potentiated by 500 nM DMI in the same manner as was the Ad_H. However, in 4 preparations, 500 nM DMI was observed to have no measurable effect on the amplitude or duration of the MCh_H elicited by a submaximal concentration of MCh (10 μM). A representative experiment is illustrated in Fig. 28. Likewise, in 4 preparations this concentration of DMI had no potentiating action on the electrically evoked slow i.p.s.p. Since only the response to exogenously applied adrenaline (Ad_H) was potentiated by 500 nM DMI and not the MCh_H nor the slow i.p.s.p., it would seem that neither of the latter two responses involve an adrenergic mechanism.

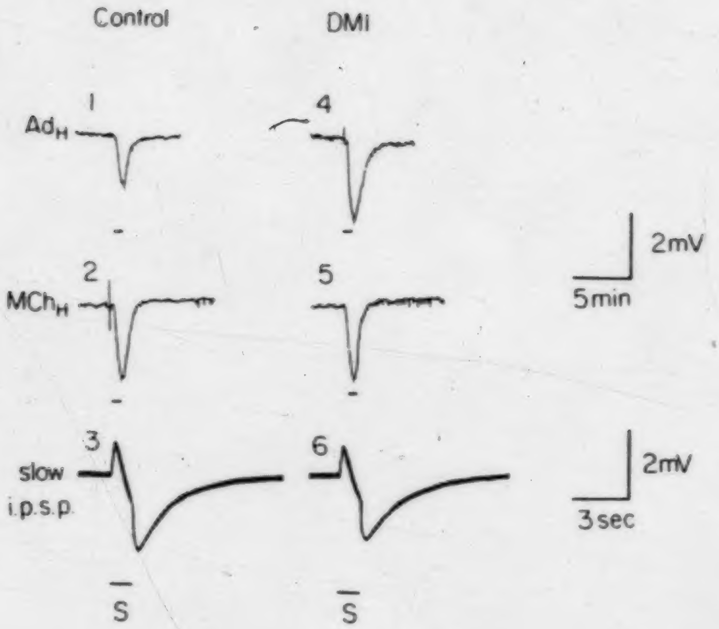


Fig. 28. Effect of DMI (desmethylimipramine) on the Ad_H, the MCh_H and the slow i.p.s.p. recorded from Rana pipiens sympathetic ganglia by means of the sucrose-gap technique. 1, 2 and 3. Control responses recorded in Ringer's solution containing 70 μM dTC. 4. Ad_H produced by 30 sec superfusion of 1 μM adrenaline recorded 10 min after starting superfusion of dTC Ringer's solution containing 500 nM DMI. 5. MCh_H evoked by a 30 sec superfusion of 10 μM methacholine and recorded after 20 min in dTC/DMI Ringer's solution. 6. Slow i.p.s.p. evoked by tetanic stimulation of VIIIth spinal nerve (1 msec pulses, 1 sec, 10 Hz) and recorded after 20 min superfusion of dTC/DMI Ringer. Note potentiation of Ad_H but not the slow i.p.s.p. or the MCh_H. Traces 1, 2, 4 and 5 from the same preparation traces 3 and 6 from another preparation. 2 mV/5 min calibration bar applies to drug responses and 2 mV/3 sec calibration to slow i.p.s.p. Black bars under responses indicate periods of superfusion with andrenaline or MCh while bar labelled s under slow i.p.s.p. indicates period of stimulation. Responses were obtained from a rectilinear pen recorder. Bandwidth was D.C. to 5 Hz for drug responses and D.C. to 15 Hz for slow i.p.s.p.'s.

3. Comparison of the sensitivities of the Ad_H, MCh_H and slow i.p.s.p. to catecholamine antagonists

a) Yohimbine

There are a number of reports in the literature claiming that yohimbine is a selective α_2-antagonist in neural tissues (for reviews see Timmermans and Van Zwieten, 1981; Goldberg and Robertson, 1983). In an earlier section of the Results, it was determined that the adrenoceptor mediating the Ad_H was most likely of the α_2-subtype. Of the antagonists tested, yohimbine was found to antagonize the Ad_H with the greatest potency (IC_{50}=6.2 nM). A low concentration of yohimbine was therefore used to test whether there was an α_2-adrenoceptor mediated component in the generation of the MCh_H and slow i.p.s.p. 100 nM yohimbine (16 times greater than the IC_{50} for antagonism of the Ad_H) failed to have any inhibitory effect on the MCh_H (to 1 mM MCh, n=4) or the slow i.p.s.p. (n=2). In the experiment illustrated in Fig. 29, the slow i.p.s.p. and MCh_H were unaltered when the Ad_H was almost completely blocked. The lack of sensitivity of the MCh_H and slow i.p.s.p. to yohimbine argues against an α_2-receptor involvement in the mediation of these response.

b) Chlorpromazine

It has been proposed that dopamine (DA) release from an interneurone serves as the neurotransmitter for the slow i.p.s.p. in mammalian ganglia (Libet, 1970). Although it has been further claimed by the same author that this DA acts on an α_2-adrenoceptor rather than a DA receptor (Ashe and Libet, 1982), it was of interest in this study

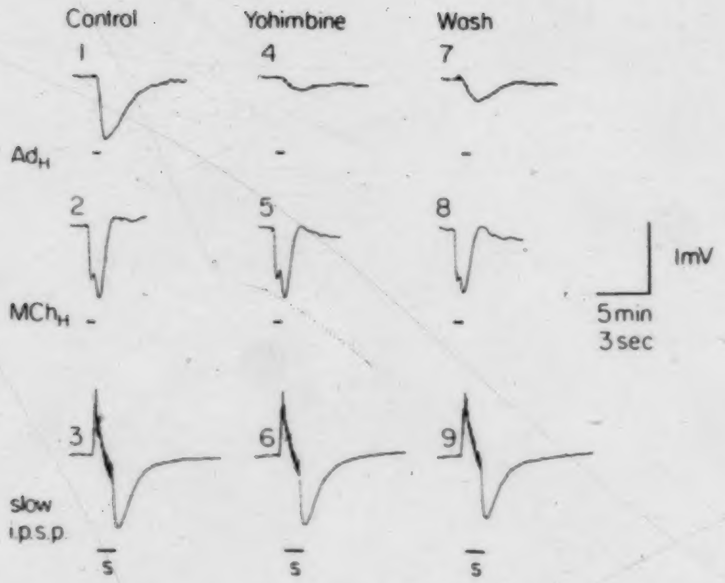


Fig. 29. Effect of yohimbine on Ad_H, MCh_H and slow i.p.s.p. from *Rana pipiens* sympathetic ganglia recorded by means of the sucrose-gap technique. All responses were obtained from the same preparation in Ringer's solution containing 500 nM DMI and 70 μM dTC. 1. Control response to 1 μM adrenaline (30 sec superfusion). 2. Control response to 1 mM MCh (30 sec superfusion). 3. Control slow i.p.s.p. evoked by stimulation of VIIIth spinal nerve (1 sec, 10 Hz, 1 msec pulse width). 4. Ad_H recorded after 35 min in 100 nM yohimbine. 5. MCh_H recorded after 50 min in 100 nM yohimbine. 6. Slow i.p.s.p. recorded after 15 min in 100 nM yohimbine. Note that Ad_H is almost blocked while the MCh_H and slow i.p.s.p. are unattenuated. Responses 7, 8 and 9 were recorded 35, 20 and 45 min after resuming superfusion with DMI/dTC Ringer's solution. 3 sec calibration bar refers to slow i.p.s.p. records only. Black bars indicate periods of superfusion of adrenaline or MCh or period of tetanic stimulation(s) for slow i.p.s.p. Traces were obtained from a rectilinear pen recorder. Bandwidth D.C. to 5 Hz for drug responses and D.C. to 15 Hz for slow i.p.s.p.'s.

to investigate whether the DA antagonist chlorpromazine (CPZ) might have an effect on the MCh_H. In four experiments, CPZ (1 μM) was found to have no effect on the MCh_H. A representative experiment is shown in Fig. 30. This figure also shows that an Ad_H (to 1 μM Ad) was slightly reduced by this equimolar concentration of CPZ. The lack of sensitivity of the MCh_H to CPZ suggests that a DA receptor is not involved in this muscarinic response. The effect of CPZ on the DA_H was not examined in these experiments because of the rapid desensitization of the DA_H. CPZ was not tested against the slow i.p.s.p. but others have shown that it was ineffective in antagonizing this response in mammalian ganglia (Dun and Karczmar, 1980).

4. Comparison of the sensitivities of the MCh_H and slow i.p.s.p. to cadmium

It is widely accepted that the evoked release (by muscarinic stimulation) of a catecholamine from a putative interneurone should be a Ca^{2+}-dependent phenomenon (Libet and Kobayashi, 1974; Weight and Smith, 1980). Therefore, if this process is involved in the generation of the slow i.p.s.p., the MCh_H should be antagonized by Ca^{2+}-channel blockers. The inorganic Ca^{2+}-channel blocker, cadmium (Cd^{2+}), has been shown to block ACh release at the frog neuromuscular junction (Cooper and Menalis, 1984). Cd^{2+} (100 μM) appeared to be effective in depressing neurotransmitter release in the *Rana pipiens* ganglia since it completely and reversibly blocked the tetanically evoked slow i.p.s.p. within 15 min in all 3 preparations examined. In contrast,

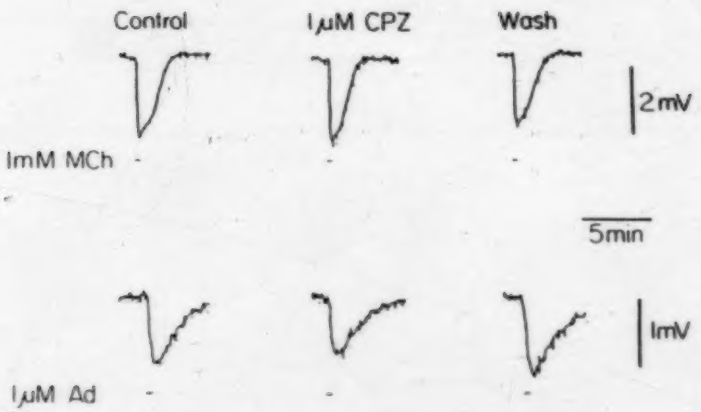


Fig. 30. Effect of chlorpromazine (CPZ) on the MCh_H and Ad_H recorded from a *Rana pipiens* sympathetic ganglion by means of the sucrose-gap technique. Upper traces: control MCh_H (to 1 mM MCh); MCh_H following 20 min exposure to 1 μM CPZ; MCh_H 10 min following removal of 1 μM CPZ. Lower traces: control Ad_H (to 1 μM Ad); Ad_H following 30 min exposure to 1 μM CPZ; Ad_H 20 min following removal of 1 μM CPZ. Black bars indicate period (30 sec) of drug superfusion. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in all Ringer's solutions.

the MCh_H (to 10 μM MCh) was never reduced but actually potentiated (to 131.9 ± 8.9% of control, n=3) following at least 15 min exposure to 100 μM Cd^{2+}. Fig. 31A indicates the time course of action of Cd^{2+} on the MCh_H and slow i.p.s.p. Some of the original sucrose-gap records of this experiment are illustrated in Fig. 31B. On the basis of these experiments, MCh would appear to produce its hyperpolarization (MCh_H) by a direct action on the primary post ganglionic neurones and not via release of a catecholamine from an adrenergic interneurone.

5. Summary - Putative catecholaminergic neurotransmission in amphibian sympathetic ganglia

Table 5 summarizes the data obtained to address this investigation. DMI (500 nM) only potentiated the response (Ad_H) to exogenously applied adrenaline, suggesting that the MCh_H and slow i.p.s.p. are direct muscarinic events. Yohimbine (100 nM) only antagonized the Ad_H, leaving the MCh_H and slow i.p.s.p. completely intact. These results again question a role for adrenaline in the MCh_H and slow i.p.s.p. Thirdly, Cd^{2+} (100 μM) selectively eliminated the slow i.p.s.p. while it had no inhibitory effect on the MCh_H. This would argue against the proposal that the generation of the MCh_H requires the release of an adrenergic neurotransmitter from an interneurone (Libet and Kobayashi, 1974). Taken together, these data favour a mechanism for direct muscarinic actions of exogenous MCh and stimulus-released ACh to produce the MCh_H and slow i.p.s.p.,

Fig. 31. Effect of 100 μM Cd^{2+} on the MCh_H and synaptic transmission (slow i.p.s.p.) in curarized Rana pipiens sympathetic ganglia recorded by means of the sucrose-gap technique. A. Graph showing time course of effect of 100 μM Cd^{2+} on slow i.p.s.p. and MCh_H. Abcissa; time in minutes, ordinate; amplitude of MCh_H or slow i.p.s.p. in mV. B. Original data records from same experiment as A. B1. Response to 30 sec superfusion of 10 μM MCh. B2. Control slow i.p.s.p. evoked by tetanic stimulation of VIIIth spinal nerve (1 sec, 10 Hz, 1 msec pulse width). B3. MCh response recorded after 35 min superfusion of dTC Ringer's solution containing 100 μM Cd^{2+}. B4. Effect of tetanic stimulation of VIIIth spinal nerve after 20 min superfusion with Ringer's solution containing 100 μM Cd^{2+}. Note blockade of slow i.p.s.p. and slight enhancement of MCh_H. B5 and 6. MCh response and slow i.p.s.p. recorded 25 and 20 min after resuming superfusion with normal dTC Ringer's solution. 5 min calibration bar refers to MCh response while 3 sec calibration bar refers to slow i.p.s.p. Black bars under responses indicate time of superfusion of MCh or period of tetanic stimulation. Traces were obtained from a rectilinear pen recorder. Bandwidth was D.C. to 5 Hz for MCh responses and D.C. to 15 Hz for slow i.p.s.p.'s. DMI (500 nM) was present in all Ringer's solutions.

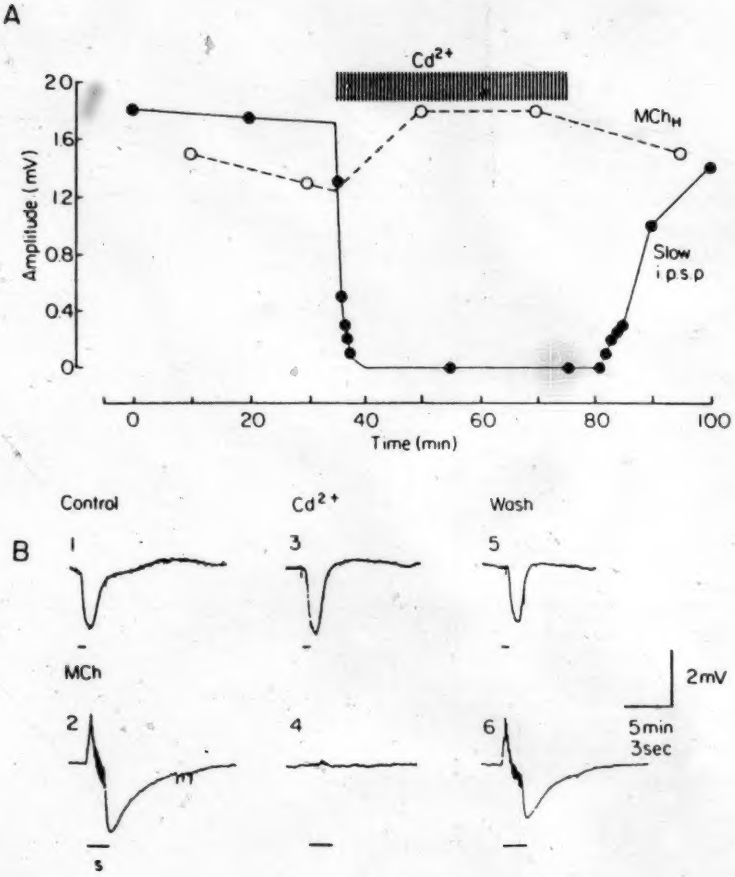

A
Cd2+
2.0
1.6
1.2
0.8
0.4
0
Amplitude (mV)
MCh_H
Slow i.p.s.p
0
20
40
60
80
100
Time (min)
B
Control
Cd2+
Wash
1
3
5
MCh
2
4
6
S
2mV
5min
3sec

Table 5

Effects of desmethylimipramine, yohimbine and cadmium on the slow i.p.s.p., MCh_H and Ad_H recorded by means of the sucrose-gap technique from Rana pipiens sympathetic ganglia.

	slow i.p.s.p.	MCh	Ad
500 nM DMI	no effect (n=4)	no effect (n=4)	169.1±4.0% (n=3)
100 nM Yohimbine	no effect (n=2)	no effect (n=4)	IC_{50}=6.2 nM (n=4)
100 μM Cd^{2+}	0.0% (n=3)	131.9±8.9% (n=3)	no effect (n=3)

a See also Results, Section A and Fig. 19 for effect of 500 nM DMI on log concentration-effect curve to adrenaline.

b See also Results, Section B and Fig. 25 for this data.

c See also Results, Section E and Fig. 43 for this data.

respectively. There would be no requirement for an adrenergic interneurone in such a mechanism.

D. Investigation of the hypothesis that adrenaline stimulates an electrogenic NaK-pump - vanadate as an electrogenic NaK-pump inhibitor

1. Ouabain and vanadate inhibition of vertebrate CNS NaK-ATPase activity

a) Bovine brain NaK-ATPase

The objective of this study was to isolate NaK-ATPase activity from a neural source as closely related as possible to frog sympathetic ganglia (i.e. the tissue in which the electrogenic NaK-pump was studied electrophysiologically) and to test the enzyme for sensitivity to vanadate. Concentration-dependent vanadate inhibition of rat brain cortical NaK-ATPase has previously been reported (Wu and Phillis, 1979). Before attempting to examine the effects of vanadate on an enzyme preparation from a neural source in frog, it was felt that the effects of vanadate should first be tested on previously established and characterized neuronal NaK-ATPase activity. Since NaK-ATPase activity had been successfully isolated from bovine brain (Rafuse et al. 1985), this enzyme was tested for its sensitivity to vanadate as a preliminary study.

A microsomal membrane preparation containing ATPase activity was made from bovine whole brain following the procedure outlined in the Materials and Methods. Spectrophotometrically, the hydrolysis of ATP was monitored by measuring the oxidative conversion of NADH to NAD^+ (see Materials and Methods). With respect to the enzyme preparation used in this study, it was found that 58.5% of the total bovine brain ATPase activity could be inhibited by 6 mM ouabain. This portion (58.5%) of the enzyme production which was sensitive to ouabain was taken to be the portion of the overall ATPase activity which represented NaK-ATPase activity. The specific activity of the ouabain-sensitive or NaK-ATPase was determined to be 18.1 μmoles Pi/mg protein/hr. The concentration-dependent effect of ouabain on the bovine brain NaK-ATPase activity is illustrated in Fig. 32A. The value for half-maximal inhibition by ouabain (IC_{50}) was estimated from the graph as shown by the broken line and was determined to be approximately 2.2 μM. The concentration-dependent inhibition of the NaK-ATPase activity by Na_3VO_4 is illustrated in Fig. 33A. Using linear regression analysis on the points between 20 and 80% of maximum inhibition, IC_{50}'s were calculated when 2 and 22 mM Mg^{2+} was present in the incubation mixture. It has been reported that elevated Mg^{2+} facilitates inhibition of NaK-ATPase by vanadate (Cantley et al. 1977). In contrast, we observed only a modest shift in the IC_{50} values from 0.69 μM (2 mM Mg^{2+}) to 0.42 μM (22 mM Mg^{2+}) in these experiments. It should be noted that these concentration-effect curves were normalized since the presence of 22 mM Mg^{2+} depressed the control level of NaK-ATPase activity (in the presence of 2 mM Mg^{2+}) by up to 30%.

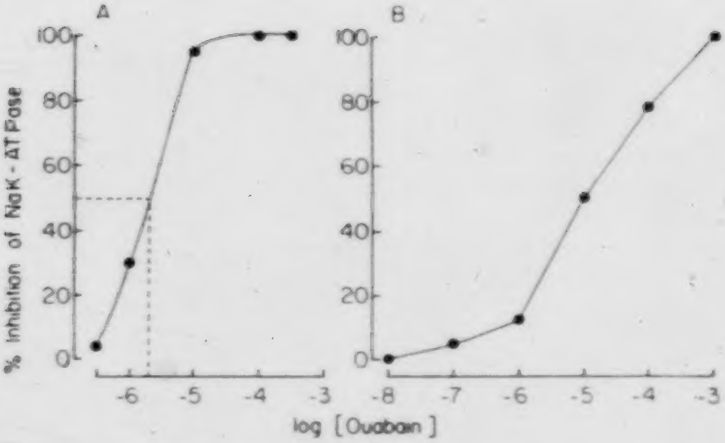


Fig. 32. A. Log concentration-effect curve of ouabain inhibition of NaK-ATPase activity isolated from bovine brain. Each point represents the mean of 3 determinations. The dashed line estimates the IC_{50} as 2.2 μM ouabain. B. Log concentration-effect curve of ouabain inhibition of NaK-ATPase activity isolated from bullfrog CNS. Each point represents the mean of 3 determinations. The IC_{50} was estimated to be 14.1 μM using the linear regression method described in the *Materials* and *Methods*.

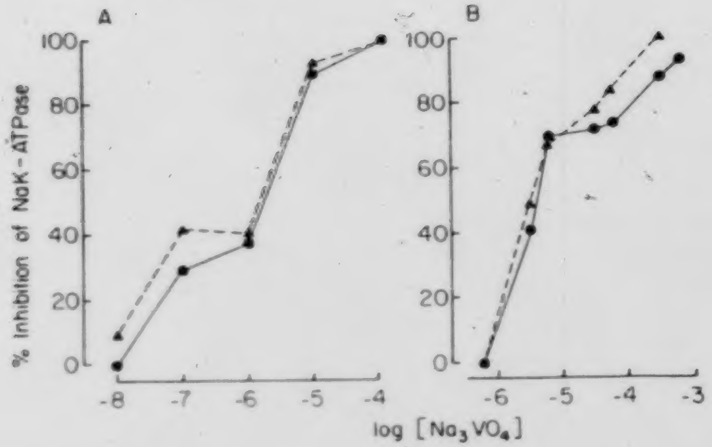


Fig. 33. A. Log concentration-effect curve of Na$_3$VO$_4$ inhibition of NaK-ATPase activity isolated from bovine brain in the presence of 2 mM Mg^{2+} (circles) and 22 mM Mg^{2+} (triangles). B. Log concentration-effect curve of Na$_3$VO$_4$ inhibition of NaK-ATPase activity isolated from bullfrog CNS in the presence of 2 mM Mg^{2+} (circles) and 28 mM Mg^{2+} (triangles). In both A and B, IC$_{50}$'s were determined by the linear regression method described in the Materials and Methods (see Table 6 and text for values). All points represent means of 3 determinations.

b) Bullfrog sympathetic ganglia ATPase

Although bovine brain provided a convenient neural source of reasonably high activity NaK-ATPase, it was the objective of this study to obtain an enzyme preparation from the same species and, if possible, tissue upon which the ensuing electrophyisological experiments were performed. Numerous unsuccessful attempts were made to procure NaK-ATPase activity from bullfrog sympathetic ganglia. The lack of success in achieving this end was undoubtably due to the extremely limited amount of tissue available. Twelve large bullfrogs yielded only 88.3 milligrams of tissue when the VIIth to Xth paravertebral sympathetic ganglia and their interconnecting sympathetic chains were removed. The method of simply homogenizing this tissue in 20 volumes of suspending solution as described in the Materials and Methods produced a slurry exhibiting no more than 1% sensitivity to ouabain. In addition, the flocculent consistency of the slurry resulted in variable amounts of enzyme being pipetted in equal volumes. Consequently, the baseline ATPase activity measured in replicate cuvettes differed considerably. This variability between assays and minimal sensitivity to ouabain precluded any NaK-ATPase studies with the sympathetic ganglia homogenate.

c) Bullfrog CNS NaK-ATPase

Although it was not possible to perform both the biochemical NaK-ATPase and electrophysiological NaK-pump studies on the same tissue (i.e. sympathetic ganglia), it was possible to isolate NaK-ATPase activity from frog brain and spinal cord. This allowed comparisons between neural tissue from the same genus. The enzyme was prepared from Rana catesbeiana while the sucrose-gap experiments were performed using ganglia from Rana pipiens.

Using the same isolation procedure as was used for the bovine brain enzyme, a microsomal ATPase preparation was made from bullfrog CNS tissue that was maximally inhibited 57.1% by 6 mM ouabain. The specific activity of the NaK-ATPase was determined to be 3.3 µmoles/mg protein/hr. NaK-ATPase activity was inhibited by increasing submaximal concentrations of ouabain as illustrated in Fig. 32B. An IC_{50} of 14.1 µM was determined from the straight line through the concentration-effect curve fitted by linear regression analysis. Similar treatment of the concentration-effect curve of Na_3VO_4 inhibition of NaK-ATPase activity (Fig. 33B) gave IC_{50}'s of 3.0 µM in the presence of 2 mM Mg^{2+} and 2.5 µM in the presence of 28 mM Mg^{2+}. 28 mM Mg^{2+} was observed to depress basal NaK-ATPase activity by 14%. It was therefore necessary to normalize the data in these graphs.

d) Summary of the effects of ouabain and vanadate on NaK-ATPase preparations

Table 6 summarizes the essential aspects of the NaK-ATPase data. It can be seen that the enzyme isolated from bovine brain was apparently 6.4 fold more sensitive to ouabain and about 4.3 fold more sensitive to vanadate (when 2 mM Mg^{2+} present) than the enzyme isolated from the bullfrog CNS. Some caution should be exercised when

Table 6

Ouabain and Na_3VO_4 sensitivity of NaK-ATPase isolated from bovine brain and *Rana catesbeiana* CNS

Enzyme Source	% of total ATPase Activity Inhibited by (6mM)	Specific NaK-ATPase Activity (μmolesPi/hr)	Ouabain IC_{50} (μM) 2mM Mg^{2+}	Na_3VO_4 IC_{50}(μM) [Mg^{2+}]		
				2 mM	22mM	28mM
Bovine Brain	58.5	18.1	2.2	0.69	0.42	
Bullfrog CNS	57.1	3.3	14.1	3.0		2.5

stating these differences since they may not reflect intrinsic differences in the enzymes. Instead, a number of extrinsic factors might serve to produce dissimilar values. First of all, the bovine brain had a higher specific activity (18.1 μmoles Pi/mg protein/hr) than the NaK-ATPase isolated from the bullfrog CNS (3.3 μmoles Pi/mg protein/hr. The greater contamination of the frog enzyme with extraneous protein and other membrane components could have retarded the inhibitory actions of ouabain and Na_3VO_4. Secondly, the assays were conducted at different temperatures. The NaK-ATPase from the mammalian source was assayed at 37°C while the NaK-ATPase from the amphibian sources was assayed at uncontrolled room temperature. It should also be noted from Table 6 that neither the vanadate inhibition of the bovine nor the frog NaK-ATPases appeared to be Mg^{2+}-dependent to any appreciable extent. This is in contrast to the findings of others (Smith et al. 1980) working with enzymes isolated from different sources.

2. Effect of vanadate on the adrenaline induced hyperpolarization

It has been established in the previous section that the biochemical expression of the electrogenic NaK-pump (i.e. NaK-ATPase) in amphibian neuronal tissue is inhibited by Na_3VO_4 in a concentration-dependent fashion. It would follow then that vanadate might serve as a useful tool to investigate the hypothesis that the Ad_H recorded by the sucrose-gap technique is produced by stimulation of the electrogenic NaK-pump (Koketsu and Nakamura, 1976). When Na_3VO_4 was applied at a concentration of 10 μM (3.3 times the IC_{50} for inhibition of bullfrog CNS NaK-ATPase) for periods of an hour or more on Rana pipiens sympathetic ganglia, no depression of the Ad_H was observed. At a concentration of 100 μM, which would have produced maximal inhibition of the isolated enzyme, a reduction of the Ad_H to 67% of control amplitude was observed after 25 min (Fig. 34A). 1 mM Na_3VO_4 caused a rapid and marked reduction to 31.6 ± 7.0% of control (n=13) after 10-20 min (Fig. 34B). Recovery of the Ad_H from this inhibitory action of 1 mM Na_3VO_4 was much slower than the rate of onset and it typically took up to 90 min before Ad_H's of control amplitude were again obtained. It would therefore appear that the Ad_H in Rana pipiens sympathetic ganglia was reduced by Na_3VO_4, but that it was much less sensitive than was the NaK-ATPase isolated from Rana catesbeiana spinal cord and brain.

Catecholamines should be oxidized by pentavalent orthovanadate (Cantley et al. 1978a; also see Fig. 4 in Introduction). The protocol of the sucrose-gap experiments demanded that the adrenaline (usually 1 μM) applied during the superfusion of orthovanadate be mixed with 1 mM Na_3VO_4. It might be expected that the overwhelming amount of vanadate (1000 times the concentration of adrenaline) would chemically inactivate the adrenaline by oxidation and thus account for the observed effect of Na_3VO_4 on the Ad_H. To test for this possibility experiments were conducted where 30 sec applications of 1 μM Ad were alternated at 15 min intervals with 30 sec applications of 1 μM Ad

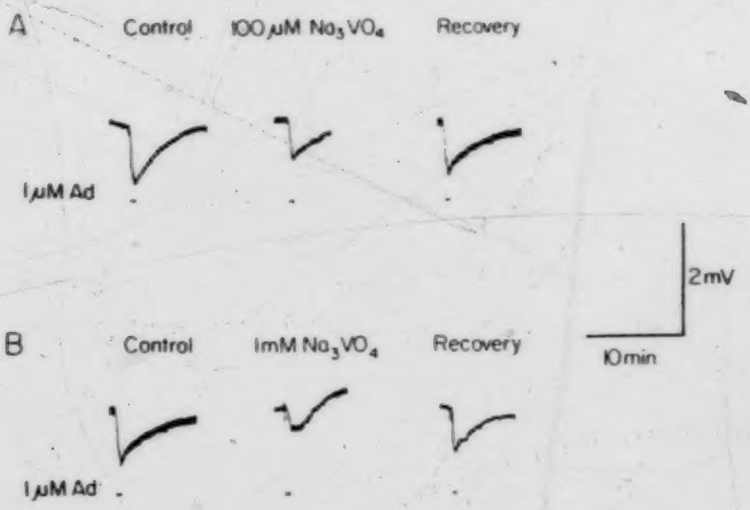


Fig. 34. Effect of Na_3VO_4 on the Ad_H recorded from *Rana pipiens* sympathetic ganglia by means of the sucrose-gap technique. A. Control Ad_H (to 1 μM Ad); Ad_H following 25 min exposure to 100 μM Na_3VO_4; recovery Ad_H following 5 min washout of 100 μM Na_3VO_4. B. Control Ad_H (to 1 μM Ad); Ad_H following 50 min exposure to 1 mM Na_3VO_4; recovery Ad_H following 30 min washout of 1 mM Na_3VO_4. Both A. and B. are from the same preparation. Black bars under the responses indicate the period (30 sec) of Ad superfusion. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in all Ringer's solutions.

plus 1 mM Na_3VO_4. The time at which the adrenaline and Na_3VO_4 were mixed was carefully noted. An experiment representative of 4 trials is illustrated in Fig. 35. Part B of this figure shows that the amplitude of the Ad_H remained essentially constant for 95 min. The amplitude of the Ad_H in the presence of Na_3VO_4 was observed to decline to 60% of control over a period of 90 min. Also, the original records illustrated in Fig. 35A show that with time the Ad_H, in the presence of Na_3VO_4, assumed a more rounded shape while the profile of the Ad_H, in the absence of Na_3VO_4, remained unchanged. If these effects were due to oxidation of the adrenaline, it occurred at a remarkably slow rate. It should be noted that the characteristic blue pigment which results when catecholamine compounds are mixed with excessive orthovanadate (Cantley et al., 1978a), was not observed in any of the 17 experiments performed when it was necessary to mix adrenaline with Na_3VO_4. It would therefore seem unlikely that the rapid inhibitory effect of 1 mM Na_3VO_4 on the Ad_H could be explained in terms of chemical inactivation of adrenaline by Na_3VO_4.

3. Effect of vanadate on the electrogenic NaK-pump in situ

a) The acetylcholine afterhyperpolarization as an electrogenic NaK-pump paradigm

In order to examine the possibility that Na_3VO_4 inhibited the Ad_H by an action on the electrogenic NaK-pump, it was necessary to ascertain the effects of Na_3VO_4 on responses known to involve

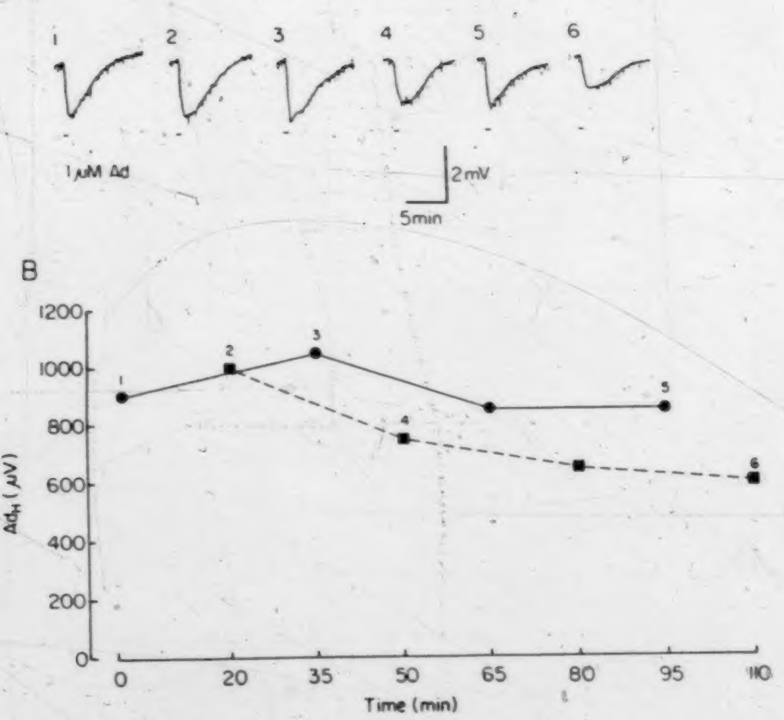


Fig. 35. Comparison of the hyperpolarization to 1 μM adrenaline in the presence and absence of 1 mM Na_3VO_4 recorded from Rana pipiens sympathetic ganglia by means of the sucrose-gap technique. A. Original traces obtained from a rectilinear pen recorder. A1. Control Ad_H (to 1 μM Ad). A2. Hyperpolarization to 1 μM Ad pre-mixed with 1 mM Na_3VO_4 for 10 min. A3. Ad_H (to 1 μM Ad). A4. Hyperpolarization to 1 μM Ad pre-mixed with 1 mM Na_3VO_4 for 100 min. A5. Ad_H (to 1 μM Ad). A6. Hyperpolarization to 1 μM Ad pre-mixed with 1 mM Na_3VO_4 for 100 min. Black bars under responses indicate period (30 sec) of Ad superfusion. B. Plot of amplitude (μV) of Ad_H (circles) and hyperpolarization to 1 μM Ad pre-mixed with 1 mM Na_3VO_4 (triangles) against time (min.). Numbers indicate responses in A. DMI (500 nM) was present in all Ringer's solutions.

activation of the pump. One such response is the afterhyperpolarization that follows the removal of nicotinic depolarizing agents (Brown et al. 1972; Smith and Weight, 1977).

Brief applications of 10 mM acetylcholine (ACh) produced a biphasic response recorded by the sucrose-gap technique. This high concentration of ACh was used because it is believed that a considerable amount of ACh esterase activity exists in these ganglia (Brzin et al. 1966) and no anticholinesterase agents were used in these experiments. The response was characterized by a large depolarization (2-5 mV, 60-90 sec in duration) followed by a long lasting hyperpolarization (1-3 mV, 5-10 min duration; see Figs. 36,37,39). It has been proposed that the hyperpolarization which follows the nicotinic depolarization to ACh occurs as a result of electrogenic NaK-pump stimulation (Brown et al. 1972; Smith and Weight, 1977). It has been reasoned that the influx of Na^+ which results from the nicotinic receptor induced increase in Na^+ and K^+ conductances activates the NaK-pump from the cytosolic side of the membrane to cause an electrogenic extrusion of Na^+ (see Introduction). This ACh afterhyperpolarization (ACh_{AH}) was inhibited by 10 μM ouabain (Fig. 36). The amplitude of the ACh_{AH} is directly dependent upon the amount of Na^+ influx and thus the amplitude of the initial depolarization to ACh (ACh_D). The amplitude of the ACh_D is dependent upon both the extent of nicotinic activation and the Na^+ gradient across the nerve membrane. Assuming that the same number of

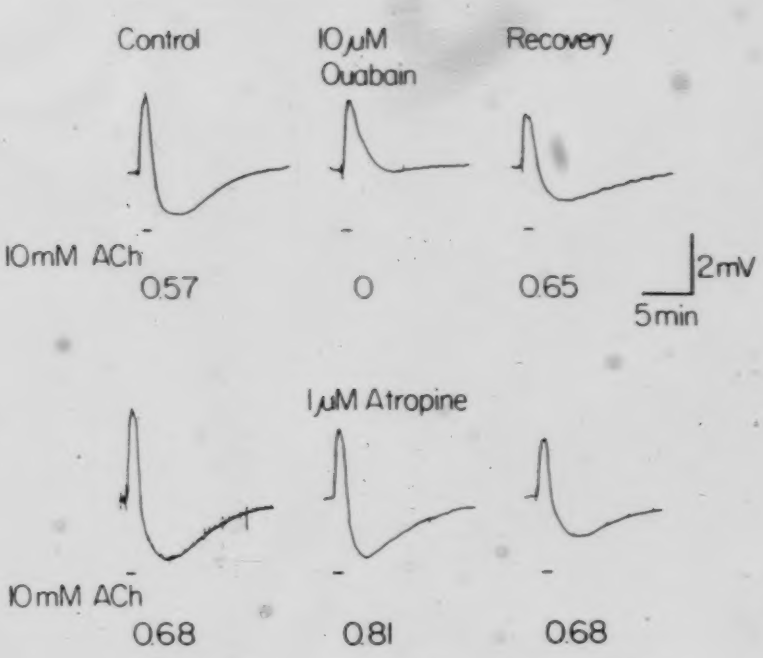


Fig. 36. Effects of ouabain and atropine on the acetylcholine (ACh) response recorded from two separate Rana catesbeiana sympathetic ganglia by means of the sucrose-gap technique. Upper traces: Control ACh response; ACh response following 47 min exposure to 10 μM ouabain; recovery ACh response following 142 min washout of ouabain. Lower traces: Control ACh response; ACh response following 32 min exposure to 1 μM atropine, recovery ACh response following 36 min washout of atropine. Black bars under responses indicate period (30 sec) of ACh superfusion. Numbers under responses indicate ACh_{AH}/ACh_D ratio. Traces were obtained from a rectilinear pen recorder.

nicotinic receptors are activated in the same way with equal applications of acetylcholine, it is reasonable to use the amplitude of the ACh_D as a rough index of the Na^+ gradient. This is an important measure to have when NaK-pump inhibitors, such as ouabain or vanadate, are applied for long periods of time. Prolonged inhibition of the pump could run down ionic gradients which could consequently affect the interpretation of the results. Due to the inextricable dependence of the ACh_{AH} on the ACh_D, the effects of various agents on the ACh_{AH} or NaK-pump have been reported as ratios of the amplitude of the ACh_{AH} to the amplitude of the ACh_D. The electrogenic NaK-pump was considered fully inhibited when this ratio became equal to zero. For example, this was achieved when the ganglion was exposed to 10 μM ouabain for 47 min in the experiment illustrated in Fig. 36.

As was mentioned in the Introduction, it has been widely observed that muscarinic agonists, such as ACh, can produce a hyperpolarization in amphibian sympathetic ganglia (Weight and Padjen, 1973; Dodd and Horn, 1983). In order to test the possibility that part of the ACh_{AH} might be due to a muscarinic receptor mediated event, 1 μM atropine was applied for 32 min (Fig. 36). This concentration of atropine did not cause a reduction and in fact usually produced a substantial increase in the ACh_{AH}/ACh_D ratio. It was therefore deemed unlikely that a muscarinic hyperpolarization contributed to the ACh_{AH} (see Discussion for possible explanation for potentiation of ACh_{AH} by atropine).

b) Effect of vanadate on the acetylcholine afterhyperolarization

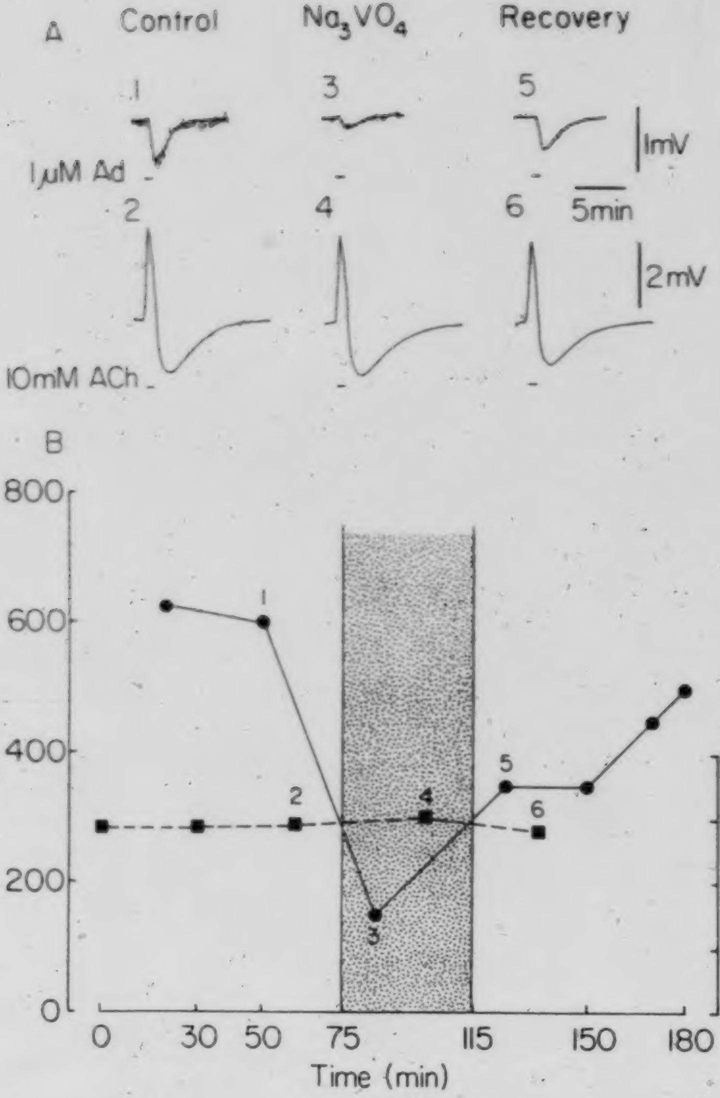
In this study, Na_3VO_4 at concentrations up to 1 mM, was superfused for up to an hour or more over 15 sucrose-gap preparations. No effect was observed on the ACh_{AH} by this exposure to Na_3VO_4 in any of these experiments. A typical experiment is illustrated in Fig. 37, where 30 sec applications of 1 μM Ad were alternated with 30 sec applications of 10 mM ACh. While the Ad_H was reduced to 25% of control in 10 min, the ACh_{AH}/ACh_D ratio remained essentially unchanged after 25 min exposure to 1 mM Na_3VO_4 (Fig. 37B). It was a consistent observation that this NaK-pump paradigm was resistant to the action of the NaK-ATPase inhibitor, orthovanadate.

c) The potassium activated hyperpolarization as an electrogenic NaK-pump paradigm

Another response believed to result from an increase in electrogenic NaK-pumping is the potassium activated hyperpolarization (K_H; Rang and Ritchie, 1968; Akasu and Koketsu, 1976).

Prolonged exposure (several hours) of isolated Rana catesbeiana sympathetic ganglia to a Ringer's solution containing 0.2 mM K^+ (1/10th the 2 mM K^+ in normal frog Ringer's solution) presumably reduced the resting NaK-pump activity in these neurones (Akasu and Koketsu, 1976). A 90 sec superfusion of Ringer's solution containing 2 mM K^+ resulted in a transient hyperpolarization (K_H, 2-5 mV; Fig. 38A). Reversal of the K_H by 1 μM ouabain supports the hypothesis that this response results from extracellular K^+ activating the inhibited electrogenic NaK-pump (Fig. 38A; also see Introduction).

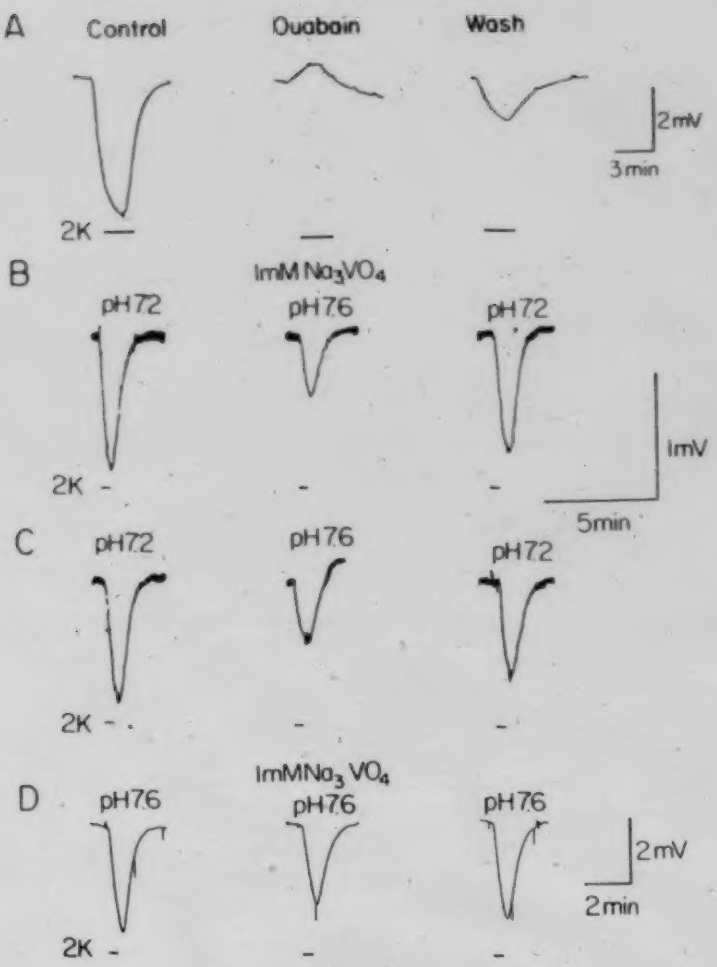
Fig. 37. Comparison of the effect of 1 mM Na_3VO_4 on the Ad_H and ACh response recorded from *Rana pipiens* sympathetic ganglia by means of the sucrose-gap technique. A. Original traces were obtained from a rectilinear pen recorder. A1. Control Ad_H (to 1 μM Ad). A2. Control ACh response (to 10 mM ACh). A3. Ad_H following 10 min exposure to 1 mM Na_3VO_4. A4. ACh response following 25 min exposure to 1 mM Na_3VO_4. A5. Ad_H following 10 min washout of 1 mM Na_3VO_4. A6. ACh response following 20 min washout of 1 mM Na_3VO_4. Black bars under responses indicate period (30 sec) of drug applications. B. Plot of amplitude of Ad_H (circles) and ACh_{AH}/ACh_D ratio (triangles) against time (min). Numbers indicate responses in A. Shaded area indicates period of exposure (40 min) to 1 mM Na_3VO_4. DMI (500 nM) was present in all Ringer's solutions.


A
Control
Na_3VO_4
Recovery
1
3
5
1μM Ad
1mV
5min
2
4
6
2mV
10mM ACh
B
800
600
400
200
0
Ad_H (μV)
0.8
0.6
0.4
0.2
0
0
30
50
75
115
150
180
Time (min)

d) Effect of vanadate on the potassium activated hyperpolarization

Following the incubation of *Rana pipiens* sympathetic ganglia in 0.2 mM K^+-Ringer's solution for several hours (overnight in some cases), normal frog Ringer's solution containing 2 mM K^+ was superfused for 30 sec at 15 min intervals. It was important that the interval of K^+ application be rigidly fixed to ensure that an equal amount of Na^+ loading occurred between each K^+ addition. Usually 4-8 K_H's had to be elicited before the responses assumed a constant amplitude. Superfusion of 1 mM Na_3VO_4 caused a depression of the K_H response to 81.3 ± 5.9% of control (n=5) in 10-15 min. A typical example is ilustrated in Fig. 38B. This effect was rapidly reversible with full recovery noted by the first K_H recorded following washout of the 1 mM Na_3VO_4. It had been noticed that the quantity of solid Na_3VO_4 required to make a 1 mM solution exceeded the buffering capacity of the TRIS Ringer's solution thus causing an elevation of the pH of the Ringer's solution from 7.2 to 7.6. Any attempts to bring the pH of the 1 mM Na_3VO_4 solution back down to pH 7.2 with acid resulted in the formation of a bright yellow solution. This color change was suspected to result from polymerization of the orthovanadate to decavanadate (Macara, 1980). To avoid this, the Na_3VO_4 Ringer's solution was routinely left at pH 7.6. To test for the effect that the difference in pH between the control Ringer's and Na_3VO_4 Ringer's solutions might have on the K_H, two 0.2 mM K^+

Fig. 38. Potassium activated hyperpolarizations (K_H's) recorded by means of a sucrose-gap technique from amphibian sympathetic ganglia, incubated in K^+-free Ringer's solution. A. Control K_H in *Rana catesbeiana* ganglion; response to 2 mM K^+ following 69 min exposure to 1 μM ouabain; recovery K_H following 199 min washout of 1 μM ouabain. pH of all solutions was 7.2. B. Control K_H to 30 sec superfusion of 2 mM K^+ recorded from *Rana pipiens* sympathetic ganglion (K^+-free and 2 mM K^+ Ringer's solution, pH 7.2); response to 2 mM K^+ following 40 min exposure to 1 mM Na_3VO_4 (in K^+-free Ringer's solution, pH 7.6); recovery K_H recorded in K^+-free Ringer's solution (pH 7.2) following 61 min washout of 1 mM Na_3VO_4. C. Control K_H to 30 sec superfusion of 2 mM K^+ recorded from same preparation as B (K^+-free and 2 mM K^+ Ringer's solution, pH 7.2); response to 2 mM K^+ following 25 min exposure to K^+-free Ringer's solution (pH 7.6); recovery K_H recorded in K^+-free Ringer's solution (pH 7.2) following 25 min washout of K^+-free Ringer's solution (pH 7.6). D. Control K_H to 30 sec superfusion of 2 mM K^+ recorded from *Rana pipiens* sympathetic ganglia (K^+-free and 2 mM K^+ Ringer's solutions, pH 7.6); response to 2 mM K^+ following 5 min exposure to 1 mM Na_3VO_4 (in K^+-free Ringer's solution, pH 7.6); recovery K_H recorded in K^+-free Ringer's solution (pH 7.6) following 65 min washout of 1 mM Na_3VO_4. Black bars under responses indicate periods of superfusion of 2 mM K^+. All traces were obtained from a rectilinear pen recorder. Middle calibration bars apply to rows B and C.


A
Control
Ouabain
Wash
2mV
3min
2K
B
1mM Na3VO4
pH7.2
pH7.6
pH7.2
1mV
5min
2K
C
pH7.2
pH7.6
pH7.2
2K
D
1mMNa3 VO4
pH7.6
pH7.6
pH7.6
2mV
2min
2K

Ringer's solutions were made up; one at pH 7.2, the other at pH 7.6. In one experiment it was observed that the K_H was rapidly and reversibly antagonized to 71% of control in 10 min by the high pH Ringer's solution (Fig. 38C). Furthermore, elevating the pH of the control Ringer's solution (with 1 M NaOH) to match that of the Na_3VO_4 Ringer's solution (both solutions at pH 7.6) allowed a demonstration that 1 mM Na_3VO_4 did not produce a statistically significant depression of the K_H in 20 min (to 94.8% of control, with 95% confidence limits of 82.8-106.8%, n=3). One of these experiments is shown in Fig. 38D. It would seem that this NaK-pump paradigm was not very sensitive to high concentrations of externally applied orthovanadate; but was instead, sensitive to solutions of basic pH. It should be noted that in contrast to the K_H, the Ad_H was not affected by solutions of basic pH.

4. Effects of anion channel blockers on the actions of vanadate on the adrenaline induced hyperpolarization

According to the hypothesis of Cantley and his colleagues (1978b), in order for catecholamines to reverse a tonic inhibition by vanadate of the NaK-ATPase, both the vanadate and adrenaline would have to gain access to the site of vanadate's action: the cytoplasmic side of the enzyme (see Introduction and Fig. 4). Therefore, it would follow that if Na_3VO_4 reduced the Ad_H by inhibiting the electrogenic NaK-pump, blockade of vanadate entry (if it occurs) into the neurones should prevent the inhibitory action of Na_3VO_4 on the Ad_H.

a) Dinitrostilbene disulphonic acid

4,4'-Dinitrostilbene 2,2'-disulphonic acid (DNDS) is an agent which reversibly blocks anion transport in human red blood cells (IC_{50}=2.0 μM; Barzilay et al. 1979) and has been reported to block erythrocyte uptake of ^{48}V (Cantley et al. 1978b). In 2 sucrose-gap experiments 50 and 150 μM DNDS failed to antagonize the inhibitory effect of 1 mM Na_3VO_4 on the Ad_H. The experiment using 50 μM DNDS is illustrated in Fig. 39. 50 μM DNDS plus 1 mM Na_3VO_4 was observed to reduce the Ad_H to 23.5% of control while 150 μM DNDS plus 1 mM Na_3VO_4 reduced the Ad_H to 33.3% (both in 10 min). In both cases, these values fall within the 95% confidence limits for the extent of depression observed with 1 mM Na_3VO_4 alone (to 31.6 (16.3-46.9)% of control, n=13). Therefore, at concentrations of DNDS which have been reported to block anion transport in red blood cells (Barzilay et al. 1979), no protection of the Ad_H from vanadate inhibition was observed.

b) Anthracene-9-carboxylic acid

Anthracene-9-carboxylic acid (A9CA) has been reported to block Cl^- conductance in skeletal muscle (Ki=11 μM; Palade and Barchi, 1977). It was reasoned that in the absence of a specific vanadate transport mechanism (such a system has not been described for any vertebrate cell type) orthovanadate might pass through the same anion channels as does Cl^-. In 3 sucrose-gap experiments, 50 μM A9CA in combination with 1 mM Na_3VO_4 was observed to produce a reduction of the Ad_H to 58.8 ± 8.8% of control in 10 min. 1 mM Na_3VO_4 alone had

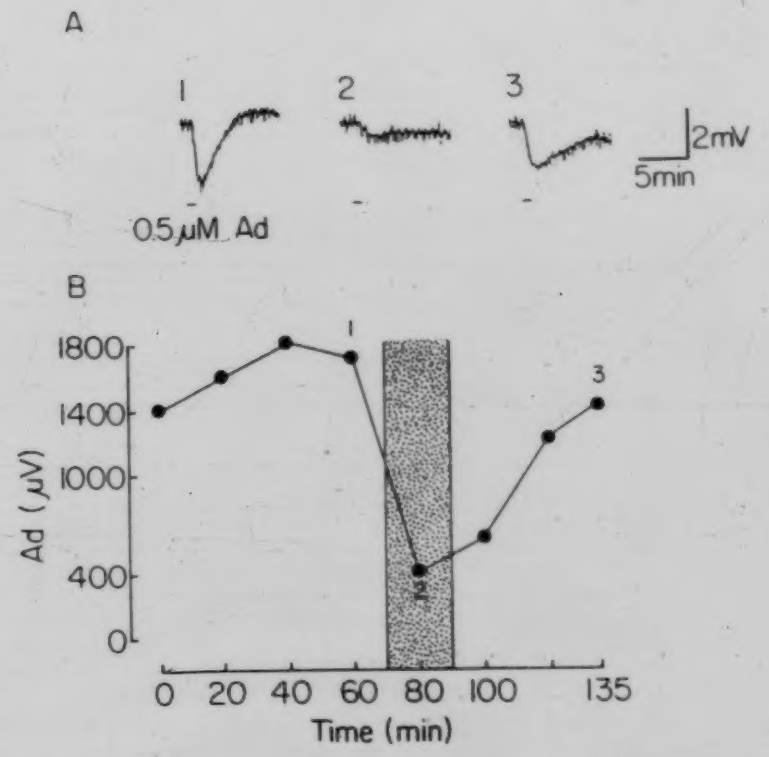


Fig. 39. Effect of DNDS on the inhibition of the Ad_H by 1 mM Na_3VO_4 recorded from a *Rana pipiens* sympathetic ganglion by means of the sucrose-gap technique. A1. Control Ad_H (to 0.5 μM Ad); A2. Response to 0.5 μM Ad following 10 min exposure to 1 mM Na_3VO_4 plus 50 μM DNDS; A3. recovery Ad_H following 45 min washout of Na_3VO_4 plus DNDS. Black bars under responses indicate period (30 sec) of superfusion of 0.5 μM Ad. Traces were obtained from a rectilinear pen recorder. B. Plot of amplitudes of Ad_H (μV) against time (min). Shaded area indicates period of exposure to 1 mM Na_3VO_4 plus 50 μM DNDS. Numbers indicate responses from A. DMI (500 nM) was present in all Ringer's solutions.

previously been observed to produce a greater degree of inhibition (to 31.6 ± 7.0% of control, n=13) but the preparation had been exposed to 1 mM Na_3VO_4 for as long as 20 min instead of 10 min as in these experiments. Fig. 40 illustrates one instance when 50 μM A9CA plus 1 mM Na_3VO_4 produced a greater inhibitory effect (to 44.4% of control) than did 1 mM Na_3VO_4 alone (to 57.5% of control). It would therefore seem that A9CA, like DNDS, did not convincingly protect the Ad_H against the inhibitory action of 1 mM Na_3VO_4. It might reasonably be suggested on the basis of these experiments that vanadate produced its inhibitory effects on the Ad_H by an external mechanism.

E. Investigation of the possible involvement of calcium in the adrenaline induced hyperpolarization

1. Adrenaline induced hyperpolarization - potassium conductance

The hypothesis that the Ad_H was produced by stimulation of the electrogenic NaK-pump was addressed in the preceeding section of the Results (for review see Kuba and Koketsu, 1978). As an alternative explanation for the electrogenesis of the Ad_H in frog sympathetic ganglia, Smith (1984) has proposed that the Ad_H results from an increase in potassium conductance (g_K). It was shown in this study that the Ad_H recorded by the sucrose-gap technique was reduced in amplitude when the $[K]_o$ was increased. Also the Ad_H was augmented in low K^+-Ringer and reversed polarity at the estimated equilibrium potential for K^+ (E_K). These findings in Rana pipiens sympathetic ganglia are in agreement with studies in other vertebrate

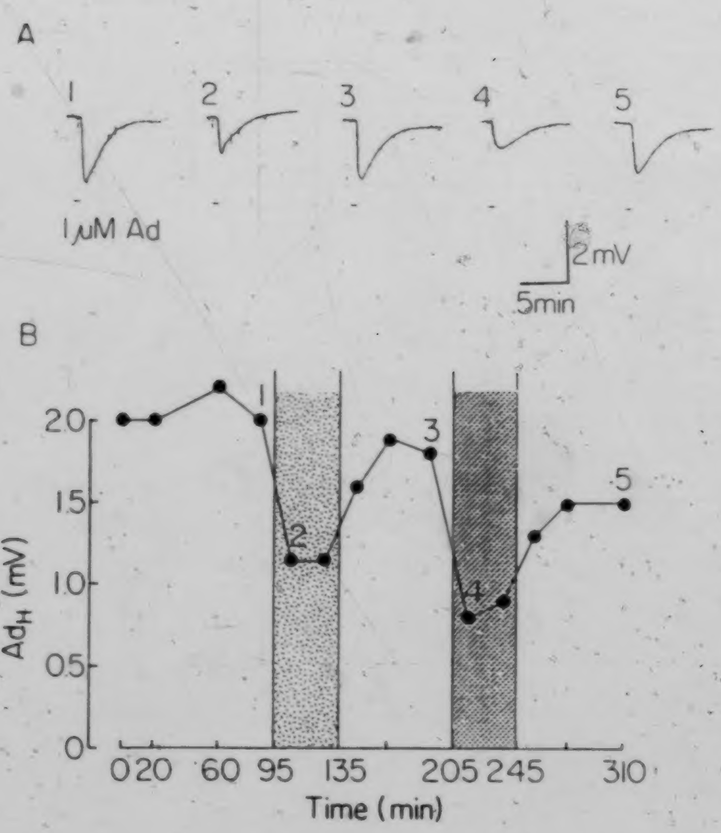


Fig. 40. Effect of anthracene 9-carboxylic acid (A9CA) on the inhibition of the Ad_H by 1 mM Na_3VO_4 recorded from a *Rana pipiens* sympathetic ganglion by means of the sucrose-gap technique. A. Original traces of a representative experiment. A1. Control Ad_H (to 1 μM Ad); A2. response to 1 μM Ad following 10 min exposure to 1 mM Na_3VO_4; A3. recovery response to 1 μM Ad following 60 min washout of 1 mM Na_3VO_4; A4. response to 1 μM Ad following 10 min exposure to 1 mM Na_3VO_4 plus 50 μM A9CA; A5. recovery response to 1 μM Ad following 65 min washout of Na_3VO_4 plus A9CA. Black bars under responses indicate period (30 sec) of superfusion of 1 μM Ad. Traces were obtained from a rectilinear pen recorder. B. Plot of amplitude of Ad_H (mV) against time (min). First shaded area indicates period of exposure to 1 mM Na_3VO_4; second shaded area indicates period of exposure to 1 mM Na_3VO_4 plus 50 μM A9CA. Numbers indicate responses in A. DMI (500 nM) was present in all Ringer's solutions.

nervous systems which suggest that α_2-adrenoceptor mediated hyperpolarizations result from an increase in gK (see Introduction).

To test further the proposal that the Ad_H results from an α_2-adrenceptor mediated increase in g_K, the effect of 2 mM Ba^{2+} on the Ad_H was investigated. Ba^{2+} blocks both voltage-sensitive (Hagiwara and Byerly, 1981; Constanti et al., 1981) and agonist activated (North and Suprenant, 1985) K^+ channels. This cation reduced the Ad_H to 11.0±6.7% of control in 30 min (n=4). The representative experiment illustrated in Fig. 41 contrasts the effects of Ba^{2+} on the Ad_H with its effects on the electrogenic NaK-pump paradigm, the ACh_{AH}. While Ba^{2+} consistently reduced the Ad_H, the ratio of the ACh_{AH} to ACh_D was markedly enhanced to 192% of control (n=4). This potentiation reflected a decrease in the ACh_D to 69.5±10.3% of control and an increase in the ACh_{AH} to 133.1±6.0% of control. Since the potential change developed by electrogenic NaK-pumping is critically dependent on the membrane resistance (Rang and Ritchie, 1968), an enhancement of the ACh_{AH} might be expected if the resting g_K was reduced in the presence of Ba^{2+}. Consequently, the potentiation of the ACh_{AH} could be explained in terms of a greater voltage response to the same amount of NaK-pump current. The marked depolarization of the resting membrane potential, which occurred when the Ringer's solution containing 2 mM Ba^{2+} was superfused over the ganglion, probably reflected a decrease in resting g_K.

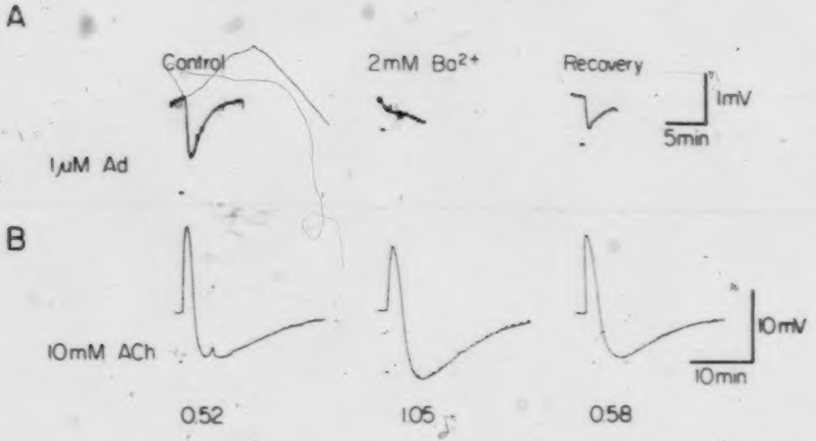


Fig. 41. Effects of Ba^{2+} on the Ad_H and ACh response recorded from a *Rana catesbeiana* sympathetic ganglion by means of the sucrose-gap technique. A. Control Ad_H (to 1 µM Ad); Ad_H completely abolished following 30 min exposure to 2 mM Ba^{2+}; recovery Ad_H following 50 min washout of Ba^{2+}. B. Control ACh response (to 10 mM ACh); ACh response following 40 min exposure to 2 mM Ba^{2+}; recovery response to 10 mM ACh following 60 min washout of Ba^{2+}. Black bars under the responses indicate period (30 sec) of drug application. Numbers under ACh responses indicate ACh_{AH}/ACh_D ratios. Traces were obtained from a rectilinear pen recorder.

2. Adrenaline induced hyperpolarization - calcium activated potassium conductance

a) Extracellular calcium

An important consideration when investigating α-receptor mediated effects of catecholamines is whether the response is dependent upon the presence of extracellular Ca^{2+} (see Introduction). Furthermore, with regard to the classification of K^+ channels in neuronal and non-neural membranes, there is an apparent dichotomy between those channels which are activated by Ca^{2+} and those which are not.

The effects of the inorganic Ca^{2+} channel blockers, Mn^{2+} and Cd^{2+}, were examined on the Ad_H using the sucrose-gap technique. It was found that 10 mM Mn^{2+} (n=4) had no effect on the Ad_H (to 10 μM adrenaline) in *Rana catesbeiana* ganglia after 15 minutes. Likewise, 100 μM Cd^{2+} (n=4) had no effect on the Ad_H (to 500 nM adrenaline in the presence of 500 nM DMI) in *Rana pipiens* sympathetic ganglia after 25 minutes. Representative experiments are illustrated in Fig. 42. These experiments would tend to suggest that extracellular Ca^{2+} is not critical for the immediate generation of the Ad_H.

b) Intracellular calcium

If the ganglion preparation was exposed to a Ca^{2+}-free Ringer's solution for prolonged periods (up to 35 min), the Ad_H was observed to slowly and reversibly decline. A typical experiment is shown in Fig. 43. Although the effect of Ca^{2+}-free Ringer on the Ad_H was not compared with its effect on a control drug response (eg. the

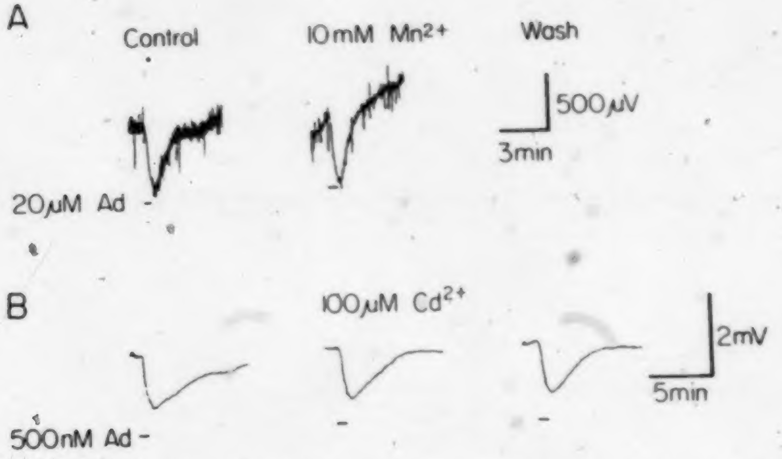


Fig. 42. Effect of divalent cations on the Ad_H recorded from *Rana pipiens* sympathetic ganglia by means of the sucrose-gap technique. A. Control Ad_H (to 20 μM Ad); response to 20 μM Ad following 17 min exposure to 10 mM Mn^{2+}. B. Control Ad_H (to 500 nM Ad); response to 500 nM Ad following 25 min exposure to 100 μM Cd^{2+}; recovery response to Ad following 10 min washout of Cd^{2+}. Experiment in B. was performed in the presence of 500 nM DMI. Black bars under responses indicate periods of Ad superfusion. Traces were obtained on a rectilinear pen recorder.

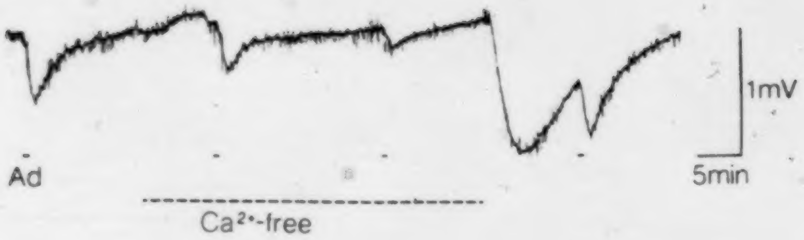


Fig. 43. Effect of Ca^{2+}-free Ringer's solution on the Ad_H (to 1 µM Ad) recorded from a Rana pipiens sympathetic ganglion by means of the sucrose-gap technique. Responses to 1 µM Ad were induced 5 and 25 min following the application of the Ca^{2+}-free Ringer's solution. A recovery Ad_H was obtained 10 min following removal of the Ca^{2+}-free Ringer's solution. Black bars under the Ad_H's indicate periods of Ad superfusion. The dashed line indicates the period of exposure to the Ca^{2+}-free Ringer's solution. Note the pronounced hyperpolarization (approx. 1 mV) resulting from the change from Ca^{2+}-free Ringer's solution to 1.8 mM Ca^{2+} (normal) Ringer's solution. Trace was obtained from a rectilinear pen recorder. DMI (500 nM) was present in all Ringer's solutions.

nicotinic ACh depolarization), these experiments might hint that extracellular Ca^{2+} is important for the maintenance of conditions necessary for the Ad_H. It is conceivable that the absence of extracellular Ca^{2+} depletes the intracellular Ca^{2+} stores and that the Ad_H is dependent on this pool of Ca^{2+} instead of extracellular Ca^{2+}. It has been proposed in vascular smooth muscle (Leitjen and Van Breeman, 1984) and rat vas deferens (Ashoori and Tomita, 1983) that the α-adrenoceptor mediated actions of catecholamines in the absence of extracellular Ca^{2+} require the release of Ca^{2+} from intracellular storage sites (also see Introduction). In order to test the applicability of this hypothesis to the Ad_H, it was necessary to compare the Ad_H to a drug response known to result from an increase in potassium conductance activated by intracellularly released Ca^{2+}. Such a response is the caffeine induced hyperpolarization introduced in the next section of the Results.

3. The caffeine induced hyperpolarization as an experimental paradigm for a drug induced calcium activated increase in potassium conductance - effects of caffeine in bullfrog ganglia

a) Caffeine induced spontaneous hyperpolarizations and slow depolarization

The spontaneous caffeine induced hyperpolarization ($sCaff_H$) was first described in bullfrog sympathetic ganglion cells by Kuba, Minota and Nishi (1972). Kuba and his associates have since presented convincing evidence that the $Caff_H$ results from an increase in potassium conductance (g_K) which is activated by intracellularly released Ca^{2+} (Kuba and Nishi, 1976; Kuba, 1980; Koketsu, et al., 1982a). The evidence that the $sCaff_H$ and evoked $Caff_H$ ($eCaff_H$) are both due to an increase in g_K included the following observations: 1) the membrane resistance decreased at the peak of the responses, 2) the $Caff_H$ reversed at the estimated E_K and 3) the amplitude of the $Caff_H$ varied inversely with changes in $[K^+]_o$. The assertion that intracellular release of Ca^{2+} is necessary to trigger these responses was supported by the observations that: 1) the $Caff_H$'s were slowly eliminated in a Ca^{2+}-free Ringer's solution, 2) intracellular injection of EGTA blocked both responses ($sCaff_H$ and $eCaff_H$) 3) dantrolene Na antagonized the $sCaff_H$. This cellular mechanism of action of caffeine in sympathetic ganglion cells is consistent with that proposed for caffeine in skeletal muscle (Weber and Herz, 1968).

In the present experiments (utilizing conventional intracellular recording techniques; see Materials and Methods), continuous superfusion of Rana catesbeiana ganglion cells with Ringer's solution containing 5 mM caffeine produced $sCaff_H$'s is in 43 of 101 cells. Since it was necessary to hold cells and maintain stable recording for at least an hour, only the larger B cells were used in these studies. It should be noted that $sCaff_H$'s, as discrete events, can only be recorded by intracellular methods from single cells. Recording techniques which measure compound potentials from a population of cells, such as the sucrose-gap technique, register the $sCaff_H$'s as small random fluctuations in the average membrane potential. This is because the $sCaff_H$'s in each neurone are not synchronized with one and another.

Although $sCaff_H$'s were recorded of all different shapes and sizes, they could be divided into four basic categories according to their profiles. Examples are given in Fig. 44. All four types of $sCaff_H$ had a large, fast initial component. This initial component was then followed by a discrete slower component (Type I; see Koketsu, et al., 1982a), a slowly decaying offset without a discrete second component (Type II), no discernible secondary phase at all (Type III), or multiple secondary fast components (Type IV). Table 7 indicates the occurence of each type of response, their total durations and the durations at half the amplitude of the initial phase of the $sCaff_H$.

The onset of action of caffeine (5 mM) is shown in Fig. 45A. In 55 of 67 cells (82%) of cells examined, the resting membrane potential (E_m) slowly depolarized and leveled off at a potential 14.2±2.1 mV less negative than the original E_m. Spontaneous $sCaff_H$'s were observed to occur after the ganglion had been exposed to caffeine for 10-30 min, depending on the flow rate of the superfusing Ringer's solution. It usually took up to another 30 min before the $sCaff_H$'s had assumed a constant amplitude and frequency. The peak amplitude of the initial phase ranged from 2-44 mV depending

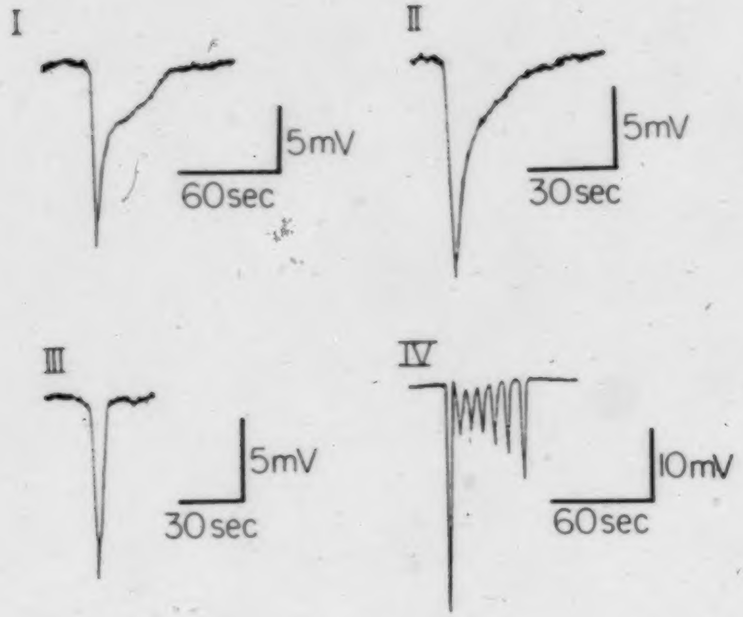


Fig. 44. Examples of four types of spontaneous caffeine (5 mM) induced hyperpolarizations (Caff$_H$) recorded intracellularly from four separate *Rana catesbeiana* sympathetic ganglion B cells. Type I sCaff$_H$'s exhibited a fast initial component followed by a slow secondary phase. Type II sCaff$_H$'s exhibited a single fast phase with a rate of onset much faster than the rate of offset. Type III sCaff$_H$'s exhibited a single fast transient phase with roughly equal onset and offset rates. Type IV sCaff$_H$'s exhibited an initial fast component (similar to a Type III sCaff$_H$) followed by several smaller fast components. Traces were obtained from a rectilinear pen recorder.

Table 7

Types of sCaff$_H$'s recorded intracellularly from B neurones of *Rana catesbeiana* sympathetic ganglia

	Types of sCaff$_H$'s			
	I	II	III	IV
Percentage of B cells which responded	$\frac{14}{43}$ = 32%	$\frac{15}{43}$ = 35%	$\frac{12}{43}$ = 28%	$\frac{2}{43}$ = 5%
Duration at 1/2 amplitude (w, sec)	12.5±3.8	5.9±0.7	3.3±0.4	3.5±0.5
Total duration (d, sec)	51.9±7.2	38.7±9.3	6.0±0.6	72.0±10.0

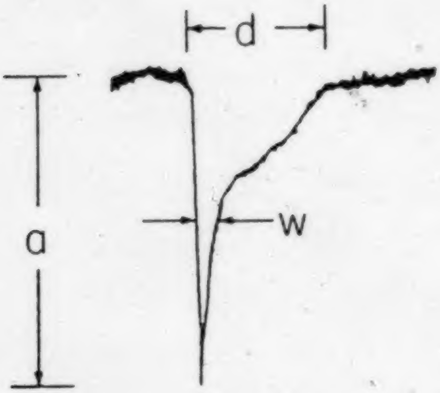
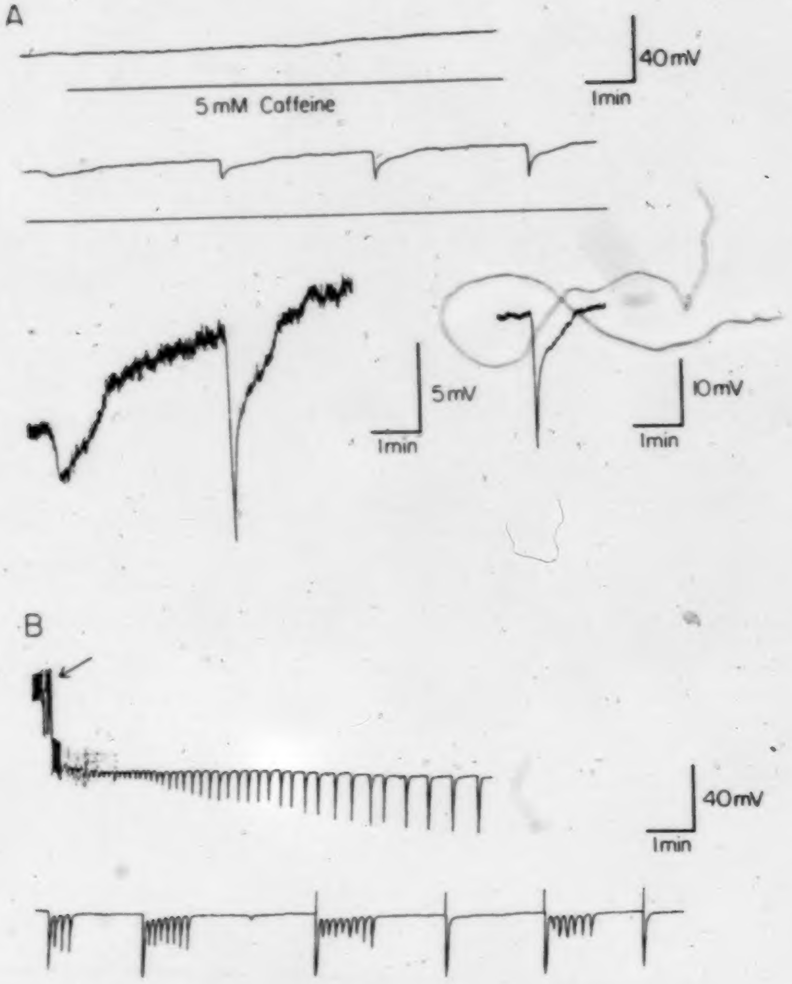


Fig. 45. Onset of caffeine actions recorded intracellularly from two *Rana catesbeiana* sympathetic ganglion B cells. A. Upper traces are a continuous low gain recording of the membrane potential of a B cell with the period of exposure to 5 mM caffeine indicated by the horizontal bars. The lower traces are the initial sCaff_H's shown in the upper traces at two higher gains. See calibration bars. Note that the resting membrane potential underwent a gradual depolarization of approximately 15 mV in response to 5 mM caffeine in this cell. B. Upper trace shows the penetration (arrow) of a B cell (which was already exposed to 5 mM caffeine. Note that the amplitudes of the Type III sCaff_H's increase with time. This probably reflects sealing of the cell membrane around the microelectrode. The lower trace indicates that the Type III sCaff_H's evolved into Type IV sCaff_H's. The final four sCaff_H's (Types III and IV) were evoked by antidromic stimulation. All traces were obtained from a rectilinear pen recorder.


A
40mV
1min
5mM Caffeine
5mV
1min
10mV
1min
B
40mV
1min

on the cell. The average amplitude of the initial phase in 43 cells was 16.5±2.0 mV. The frequency of the $sCaff_H$'s varied from 0.1-6.0 min^{-1} with an average of 1.3±0.3 min^{-1}. In many experiments, the caffeine (5 mM) was already being superfused over the ganglion at the time the cell was impaled. In these cases, small, high frequency $sCaff_H$'s were immediately noticeable and these were observed to increase in amplitude and decrease in frequency with time until they became constant. It is likely that this progressive change reflects recovery from penetration injury caused by the electrode. An example of this is shown in Fig. 45B.

Although the slow initial depolarization to caffeine has been reported to increase in amplitude with concentration (Kuba and Nishi, 1976), the amplitudes of the $sCaff_H$'s were not found to be concentration-dependent. Lower concentrations of caffeine were observed to produce $sCaff_H$'s in a lower percentage of the neurones examined. In the present experiments, 5 mM caffeine was used throughout to optimize the likelihood of inducing $sCaff_H$'s (48 of the 101 B cells examined produced $sCaff_H$'s).

b) Evoked caffeine induced hyperpolarizations

Hyperpolarizations were electrically evoked in the presence of 5 mM caffeine ($eCaff_H$'s) in 40 of 49 cells (82%) examined. $eCaff_H$'s could often be evoked in cells which did not produce $sCaff_H$'s. Evoked $Caff_H$'s could be achieved by several means including orthodromic or antidromic stimulation, or by direct stimulation with depolarizing or hyperpolarizing current pulses. Examples of $eCaff_H$'s produced by direct depolarizing current injection are shown in Fig. 46A. Part B of this figure also illustrates a plotted record from the Nicolet 2090 Digital Oscilloscope showing the effect of caffeine on the AP_{AH} of an antidromically evoked B spike. This is the same general phenomenom as shown in Fig. 46A but at a faster sweep speed. Evoked $Caff_H$'s ranged in amplitude from 2 to 50 mV with an average of 17.2±1.9 mV (n=40). It should also be noted from Fig. 46A that following an $eCaff_H$, the cell was refractory for 2-4 min before another response could be evoked. The measureable features of the $Caff_D$, $sCaff_H$ and $eCaff_H$ are summarized in Table 8.

4. Comparison of the sensitivities of the Ad_H and $Caff_H$'s to potassium channel blockers

If the Ad_H involves an increase in gK activated by intracellular Ca^{2+}, then the adrenaline activated channels may have the same pharmacological properties as the Ca^{2+} activated gK which generates the $sCaff_H$ and $eCaff_H$. Therefore, the effects of various K^+ channel blockers on the Ad_H and the $Caff_H$ were compared.

a) Tetraethylammonium

Tetraethylammonium (TEA) has been shown to block two voltage-dependent gK's associated with the repolarization of the action potential in bullfrog sympathetic ganglion cells: the delayed rectifier current and the Ca^{2+}-activated gK (Adams et al. 1982). If the Ad_H is due to the activation of such voltage-dependent gK's, it

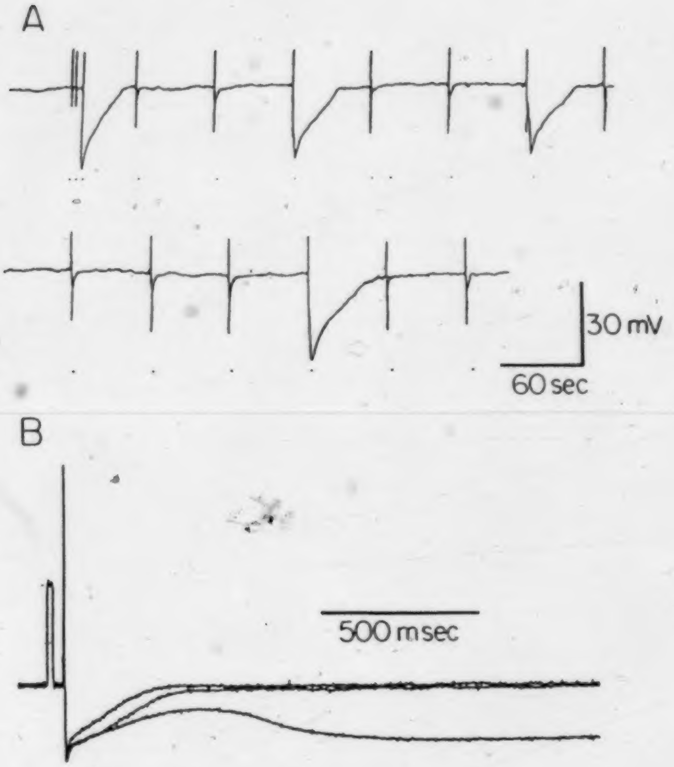


Fig. 46. Evoked caffeine (5 mM) induced hyperpolarizations (eCaff$_H$'s) recorded intracellularly from two *Rana catesbeiana* sympathetic ganglion B cells. A. Caff$_H$'s evoked by direct depolarizing current injection. Dots under continuous traces indicate times (60 sec interval) of current injection. Note that the cell appeared to be refractory or unresponsive to stimulation for 3-4 minutes following an eCaff$_H$. B. Effect of 5 mM caffeine on the antidromically evoked AP$_{AH}$ of a B cell. Exposure to 5 mM caffeine for 10 min increased the duration of the AP$_{AH}$ while exposure for 13 min resulted in an eCaff$_H$. Note time calibration in B. compared to A.. The voltage calibration immediately preceeding the antidromically evoked action potential measured 50 mV and 20 msec. A. and B. represent two separate B cells. Traces in A. were obtained from a rectilinear pen recorder. Traces in B. were obtained from a digital storage oscilloscope.

Table 8

Some characteristics of caffeine responses recorded intracellularly from B cells of *Rana catesbeiana* sympathetic ganglia

	Caff_D	sCaff_H	cCaff_H
Percentage of cells which responded	$\frac{55}{67} = 82\%$[a]	$\frac{43}{101} = 43\%$[b]	$\frac{40}{49} = 82\%$[c]
Amplitude (range)	14.2±2.1mV[d] (2-28mV)	16.5±2.0mV[e] (2-44mV)	17.2±1.9mV (2-50mV)
Frequency of sCaff_H (range)		$1.3 \pm 0.3\text{min}^{-1}$ $(0.1\text{-}6.0\text{min}^{-1})$	

a Caff_H - percentage of cells which depolarized to the application of 5 mM caffeine after the cell was impaled

b sCaff_H - percentage of cells which exhibited spontaneous hyperpolarizations to 5 mM caffeine applied either before or after the cell was impaled

c eCaff_H - percentage of stimulated cells which hyperpolarized when stimulated by any of the following methods: 1) antidromically evoked spike, 2) orthodromically evoked spike, 3) direct depolarizing current injection or 4) anodal break excitation

d Caff_D - change in membrane potential from pre-caffeine level to plateau of new potential following caffeine action

e sCaff_H - amplitude of initial fast phase of all four types of sCaff_H

might be expected that it would be blocked by TEA. TEA (10 mM) was found to produce variable effects on the Ad_H recorded by the sucrose-gap technique in Rana catesbeiana ganglia. In 5 of 8 experiments, 10 mM TEA destabilized the resting sucrose-gap potential to the extent that subsequent Ad_H's were obscured. In the remaining 3 experiments, the Ad_H was enhanced, reduced and unchanged in separate preparations. The net effect after 30 minutes exposure was not significant (98.3±15.6% of control). Fig. 47A indicates an experiment when the Ad_H was slightly potentiated after 45 minutes.

The effect of TEA on the intracellularly recorded $sCaff_H$ from individual B cells was more straight forward. In all 4 cells studied a large depolarization (20-40 mV) occurred when 10 mM TEA was applied (Figs. 48 and 49). In 2 cells this depolarization was sufficient to bring the cell to threshold and induce spontaneous activity (Fig. 49). The membrane potential returned to the pre-TEA control level and the spontaneous firing ceased when the TEA was removed. During the plateau of the TEA induced depolarization, the fast, initial component of the $sCaff_H$'s were reduced to 39.6±8.0% of control (n=4) while the secondary H's were unaffected (Fig. 48, Type I $sCaff_H$; Fig. 49, Type IV $sCaff_H$). This is consistent with the findings of Koketsu, et al., (1982a). This depressant effect may have been largely due to the depolarization itself, since it was observed in 2 experiments that injected depolarizing current, which displaced the membrane potential by the same amount as did 10 mM TEA, also reduced the amplitude of the

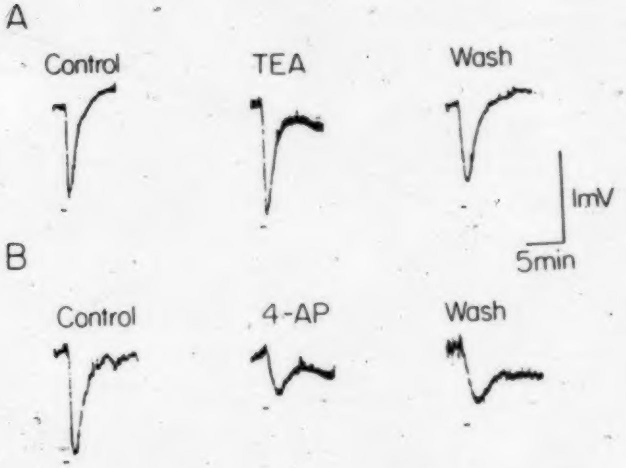


Fig. 47. Effects of TEA and 4-AP on the Ad_H recorded from two separate Rana catesbeiana sympathetic ganglia by means of the sucrose-gap technique. A. Control Ad_H (to 1 μM Ad); Ad_H following 45 min exposure to 10 mM TEA; Ad_H following 45 min washout of TEA. B. Control Ad_H (to 1 μM Ad); Ad_H following 50 min exposure to 1 mM 4-AP; recovery of Ad_H following 145 min washout of 4-AP. Black bars under responses indicate period (30 sec) of Ad superfusion. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in both experiments.

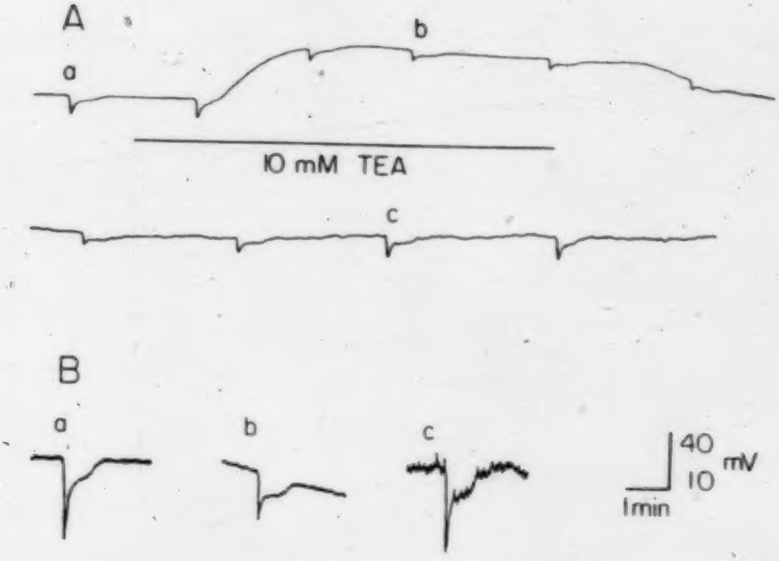


Fig. 48. Effect of TEA on Type I sCaff$_H$ recorded intracellularly from a *Rana catesbeiana* sympathetic ganglion B cell. A. Low gain continuous record of membrane potential of a B cell in the presence of 5 mM caffeine and 10 mM TEA. Caffeine was continuously present whereas 10 mM TEA was superfused for the period indicated by the horizontal bar. Note the selective effect of TEA on the initial fast component of the Type I sCaff$_H$ and the reversible depolarization produced by 10 mM TEA. B. High gain records of sCaff$_H$'s before, Ba; during, Bb; and after, 10 mM TEA, Bc. Lower case letters indicate corresponding responses in A. 40 mV calibration applies to A; 10 mV calibration applies to B. Traces were obtained from a rectilinear pen recorder.

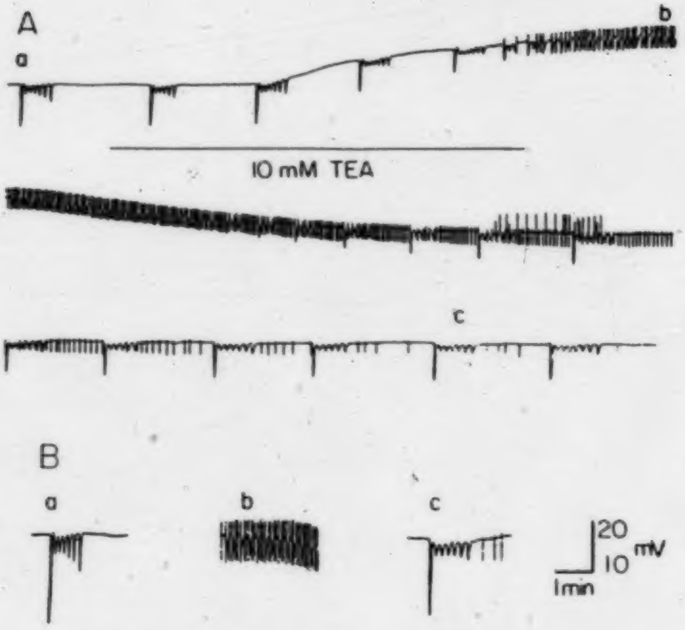


Fig. 49. Effect of TEA on Type IV sCaff$_{H}$ recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. A. Low gain continuous record of membrane potential of a B cell in the presence of 5 mM caffeine and 10 mM TEA. Caffeine was continuously present whereas 10 mM TEA was applied for the period indicated by the horizontal bar. Note the selective effect of TEA on the initial fast component of the Type IV sCaff and the reversible depolarization produced by 10 mM TEA. In this experiment the TEA induced depolarization was sufficient to bring the cell to threshold and cause spontaneous firing. This firing was also reversible with the repolarization of the membrane potential. B. High gain records of sCaff$_{H}$'s before, Ba; during, Bb; and after, Bc 10 mM TEA. Lower case letters indicate corresponding responses in A. 20 mV calibration applies to A; 10 mV calibration applies to B. Traces were obtained from a rectilinear pen recorder.

primary component of the $sCaff_H$'s to the same extent.

b) 4-aminopyridine

4-aminopyridine (4-AP) has been reported to block a fast, transient voltage-dependent gK (A-current) in a variety of invertebrate (Thompson, 1977) and vertebrate neurones (Belluzzi et al., 1985), yet has little effect on any of the K^+ channels previously described in bullfrog sympathetic ganglia (Adams, et al., 1982). Despite this, 1 mM 4-AP reversibly reduced the Ad_H to 17.2±5.4% of control after 30 min (n=5). A representative experiment is illustrated in Fig. 47B.

In contrast, 1 mM 4-AP had no effect on any phase of the $sCaff_H$'s (n=8). It was also observed that 1 mM 4-AP produced no change in the resting membrane potential (in the presence or absence of 5 mM caffeine) and it produced no change in the profile of the antidromically evoked action potential (in the absence of caffeine). These observations might suggest that 4-AP sensitive channels are not involved in the generation of an action potential or maintenance of the resting membrane potential of these cells. However, in every intracellular experiment performed with 4-AP in the presence of caffeine, spontaneous firing resulted which reached a peak frequency of about 2 Hz. A typical experiment is illustrated in Fig. 50. These spontaneous action potentials often evoked $eCaff_H$'s but the amplitude of these responses did not appear to be any smaller than the $sCaff_H$'s recorded before the application of 4-AP. It should be

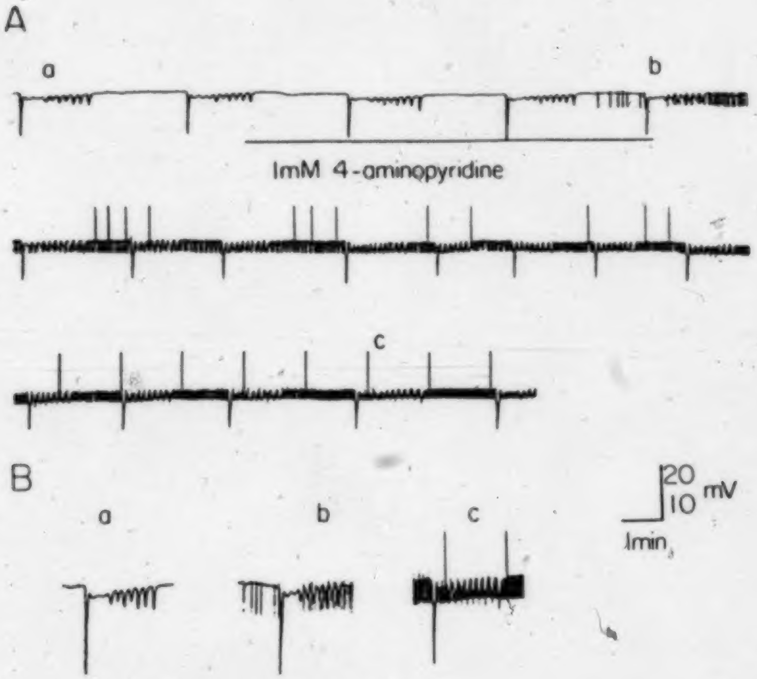


Fig. 50. Effect of 4-AP on sCaff$_H$ recorded intracellularly from a *Rana catesbeiana* sympathetic ganglion B cell. A. Low gain continuous record of membrane potential of a B cell in the presence of 5 mM caffeine and 1 mM 4-AP. Caffeine was continuously present whereas 1 mM 4-AP was superfused for the period indicated by the horizontal bar. Note the induction of irreversible spontaneous firing by 4-AP without any effect on the amplitude of any phase of the sCaff$_H$. B. High gain records of sCaff$_H$'s before, Ba; during, Bb; and after, Bc 1 mM 4-A). Lower case letters indicate corresponding responses in A. 20 mV calibration applies to A; 10 mV calibration applies to B. Traces were obtained from a rectilinear pen recorder.

noted that the induction of this spontaneous activity appeared to be an irreversible phenomenon since it continued unabated for up to 2 hours following washout of the 4-AP. Examination of the spontaneous activity on the Nicolet 2090 Digital Oscilloscope revealed that a mixture of pre- and postsynaptically generated action potentials contributed to this activity (Fig. 51). Since presynaptically generated action potentials were superimposed upon fast e.p.s.p.'s, the normal profile of the spike afterhyperpolarization was obscured and the spike amplitude was reduced. The reduction in spike amplitude was presumably due to the shunting effect of the fast e.p.s.p. (produced by an increase in Na^+ and K^+ conductance; see Section A4a of the Introduction; Blackman, et al., 1963).

c) Quinidine

Quinidine has been shown to exhibit properties as a Ca^{2+} activated gK blocker similar to its levorotary isomer quinine (Cook and Haylett, 1985). Quinine blocks the Ca^{2+} activated K^+ permeability of red blood cells (Lew and Ferreira, 1978), hepatocytes (Banks et al. 1979) and the Ca^{2+} activated gK of myenteric neurones (Cherubini et al. 1984). If the Ad_H in bullfrog ganglia involves the activation of a Ca^{2+} activated K^+ channel similar to the quinine sensitive channel described in these other cell types, quinidine might be expected to block the Ad_H.

Quinidine (50 μM) was found to reversibly reduce the amplitude of the Ad_H to 37.6±10.6% of control after 15 min in 6 sucrose-gap

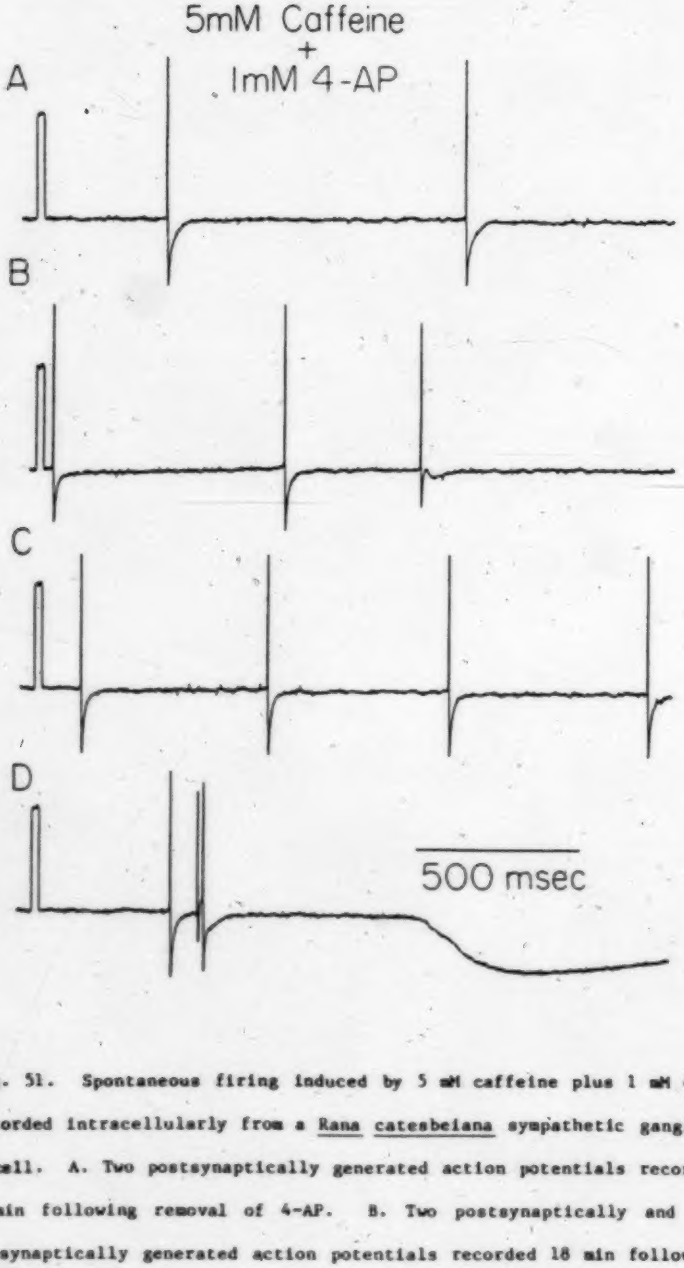


Fig. 51. Spontaneous firing induced by 5 mM caffeine plus 1 mM 4-AP recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. A. Two postsynaptically generated action potentials recorded 6 min following removal of 4-AP. B. Two postsynaptically and one presynaptically generated action potentials recorded 18 min following removal of 4-AP. C. Four postsynaptically generated action potentials recorded 24 min following removal of 4-AP. D. One presynaptically generated action potential between two postsynaptically generated action potentials followed by a $sCaff_H$ recorded 25 min after the removal of 4-AP. Voltage calibration on the left of each trace measured 50 mV, 20 ms. Traces were obtained from a digital storage oscilloscope.

experiments. A representative experiment is illustrated in Fig. 52A. Both the $sCaff_H$ and $eCaff_H$ were antagonized by 50 μM quinidine. The $sCaff_H$ was reversibly reduced to 58.1±2.0 % of control (n=6) following 15 min and the $eCaff_H$ was reversibly reduced to 37.5% of control after 10 min in one experiment. A typical experiment, indicating the inhibitory effects of quinidine on the $sCaff_H$ is illustrated in Fig. 53. The single experiment performed on the $eCaff_H$'s is illustrated in Fig. 54. Quinidine was only tested against Type II and Type III $sCaff_H$'s, therefore it was not determined whether this K^+ channel blocker had any differential effects on the initial and secondary phases. The application of 50 μM quinidine (in the presence of caffeine) was not observed to cause any effect on the resting membrane potential of these cells.

d) Apamin

The bee venom peptide, apamin, has previously been reported to block the noradrenaline induced increase in K^+ permeability of hepatocytes (Banks et al. 1979), a voltage-dependent current in rat superior cervical ganglion cells (Galvan and Behrends, 1985) as well as a slow Ca^{2+}-sensitive gK (I_{AHP}) in bullfrog sympathetic neurones (Pennefather, et al., 1985). It does not however block Ca^{2+} activated K^+ channels in all cell types. Notably, it has no effect on the Gardos current in erythrocytes (Banks et al. 1979) nor the early, voltage sensitive Ca^{2+}-sensitive gK in bullfrog sympathetic neurones. It was of interest in the present study to investigate whether apamin

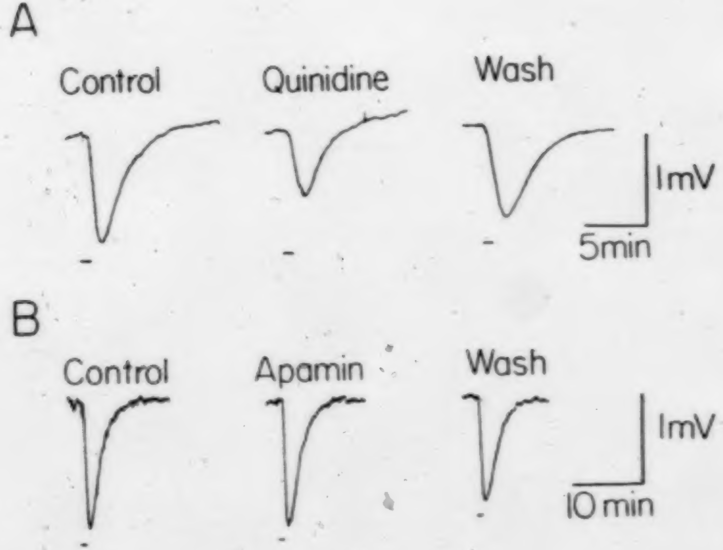


Fig. 52. Effects of quinidine and apamin on the Ad_H recorded from two separate *Rana catesbeiana* sympathetic ganglia by means of the sucrose-gap technique. A. Control Ad_H (to 1 μM Ad); Ad_H following 15 min exposure to 50 μM quinidine; recovery of Ad_H following 200 min washout of 50 μM quinidine. B. Control Ad_H (to 1 μM Ad); following 45 min exposure to 500 nM apamin; Ad_H following 80 min washout of 500 nM apamin. Black bars under responses indicate period (30 sec) of Ad superfusion. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in both experiments.

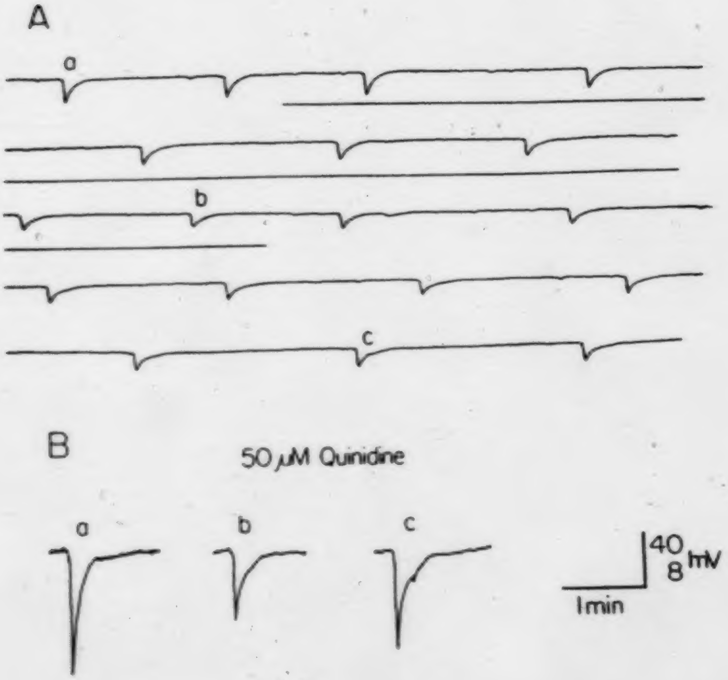


Fig. 53. Effect of quinidine on sCaff$_H$ recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. A. Low gain continuous record of membrane potential of a B cell in the presence of 5 mM caffeine and 50 μM quinidine. Caffeine was continuously present whereas 50 μM quinidine was superfused for the period indicated by the horizontal bars. B. High gain records of sCaff$_H$'s before, Ba; during, Bb; and after, 50 μM quinidine, Bc. Lower case letters indicate corresponding responses in A. 40 mV calibration applies to A; 8 mV calibration applies to B. Traces were obtained from a rectilinear pen recorder.

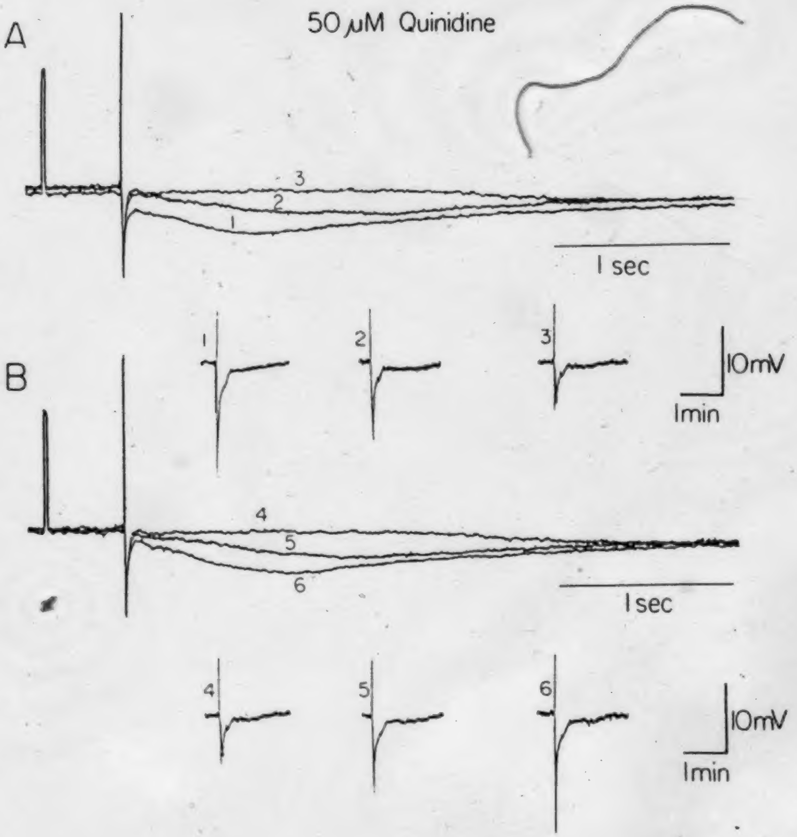


Fig. 54. Effect of quinidine on eCaff$_H$ induced by an antidromically evoked action potential recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. A. Superimposed traces from digital storage oscilloscope showing onset of quinidine action. A1. control Caff$_H$; A2. eCaff$_H$ following 7 min exposure to 50 μM quinidine; A3. eCaff$_H$ 3 min following removal of 50 μM quinidine. The same traces obtained from a rectilinear pen recorder are also shown. Note different time calibrations. B. Superimposed traces from digital storage oscilloscope showing offset of quinidine action. B1. eCaff$_H$ 3 min following removal of 50 μM quinidine; B2. 11 min following removal of quinidine; B3. 29 min following removal of quinidine. The same traces obtained from a rectilinear pen recorder are also shown. Note different time calibrations. Voltage calibration preceeding antidromically evoked action potential measures 50 mV, 20 msec.

would affect the Ad_H and $Caff_H$ in similar or different manners.

Special care was taken to ensure that the apamin used in the sucrose-gap and intracellular experiments was biologically active by assaying the peptide on rabbit jejunum (see Materials and Methods). No effect of apamin (500 nM) was observed in any of the 7 sucrose-gap experiments performed on bullfrog ganglia. A typical experiment is shown in Fig. 52B. In contrast, it was observed that 500 nM apamin appeared to selectively and reversibly antagonize the fast primary component of the $sCaff_H$ and $eCaff_H$ in two separate intracellular experiments. These experiments are illustrated in Fig. 55. It would therefore seem possible, on the basis of these few experiments, that apamin, at least partially, inhibits the Ca^{2+}-activated gK induced by caffeine while having no effect on the Ad_H.

5. Comparison of the sensitivites of the Ad_H and $Caff_H$'s which affect intracellular calcium movements

a) Dantrolene

Dantrolene is generally believed to inhibit the release of Ca^{2+} from the sarcoplasmic reticulum of skeletal muscle cells (Van Winkle, 1976). Kuba (1980) has reported that dantrolene Na (6.4 - 20 μM) either lengthened the interval between the $sCaff_H$'s or abolished them altogether. This observation was used by Kuba to strengthen his hypothesis that the $Caff_H$'s result from an increase in gK which is activated by intracellularly released Ca^{2+}.

If the Ad_H was generated in a similar fashion, it should

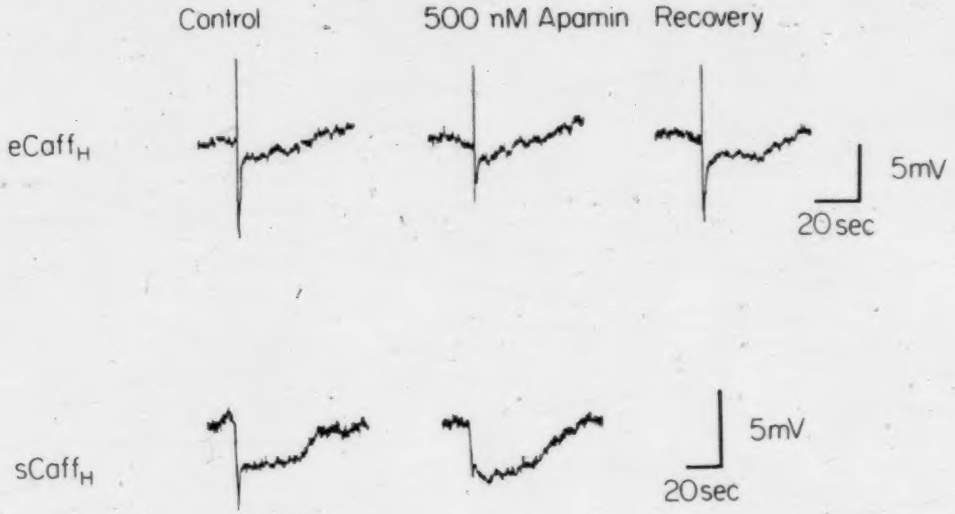


Fig. 55. Effect of apamin on eCaff$_H$ and sCaff$_H$ recorded intracellularly from two Rana catesbeiana sympathetic ganglion B cells. Upper traces show Caff$_H$'s induced by antidromically evoked action potential: control eCaff$_H$; eCaff$_H$ following 6 min exposure to 500 nM apamin; recovery eCaff$_H$ 46 min following removal of apamin. Lower traces show control sCaff$_H$ and sCaff$_H$ following 2 min exposure to 500 nM apamin. Note that only the inital fast phase of the eCaff$_H$ and sCaff$_H$ was affected by 500 nM apamin. Traces were obtained from a rectilinear pen recorder.

exhibit a similar sensitivity to dantrolene. It was therefore of interest to examine whether this compound affected the Ad_H. It was found however that 60 μM dantrolene Na had effect on the Ad_H after 45 min of superfusion in 7 sucrose-gap preparations. A typical experiment is shown in Fig. 56A.

b) 8-(N,N-diethylamino)octyl 3,4,5 trimethoxybenzoate

The compound, 8-(N,N-diethylamino)octyl, 3,4,5 trimethoxybenzoate (abbreviated as TMB-8), which is described as an "intracellular Ca^{2+} antagonist" has been shown to block caffeine induced release of Ca^{2+} bound to intracellular storage sites in various smooth and skeletal muscles (Chiou and Malagodi, 1975). In an effort to examine the role of intracellular Ca^{2+} movements in bullfrog sympathetic ganglion cells, it was felt that it would be worthwhile to test and compare the effects of TMB-8 on the Ad_H and $Caff_H$'s.

In 7 sucrose-gap preparations, TMB-8 (50 μM) was found to reversibly antagonize the Ad_H to 38.9±7.1% of control after 15 min. A representative experiment is illustrated in Fig. 56B. The same concentration of TMB-8 was found to have no effect whatsoever on the amplitude or frequency of the $sCaff_H$'s recorded in 6 cells. A typical cell is shown in Fig. 57. The amplitudes of the $eCaff_H$'s in two cells were also observed to be unaffected by 50 μM TMB-8. However, as is illustrated in Fig. 58, the latency of onset of the $eCaff_H$ following the antidromically evoked spike was reversibly increased in TMB-8. These results might suggest that TMB-8 elevates

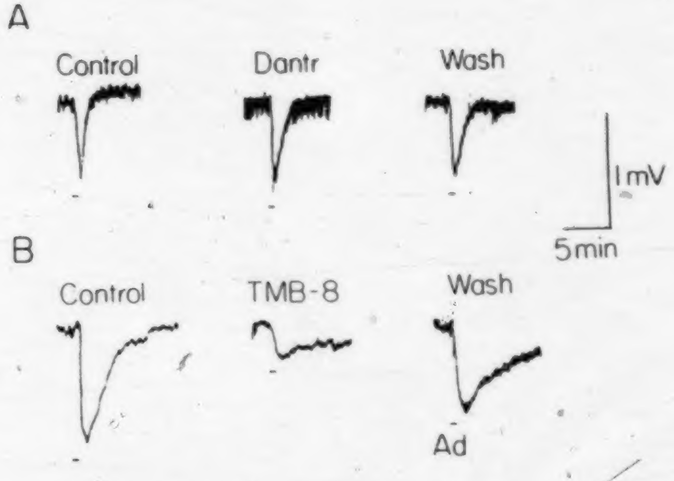


Fig. 56. Effects of dantrolene and TMB-8 on the Ad_H recorded from two separate Rana catesbeiana sympathetic ganglia by means of the sucrose-gap technique. A. Control Ad_H (to 1 μM Ad); Ad_H following 45 min exposure to 60 μM dantrolene; Ad_H following 45 min washout of dantrolene. B. Control Ad_H (to 1 μM Ad); Ad_H following 20 min exposure to 50 μM TMB-8; recovery Ad_H following 105 min washout of TMB-8. Black bars under responses indicate period (30 sec) of Ad superfusion. Traces were obtained from a rectilinear pen recorder. DMI (500 nM) was present in both experiments.

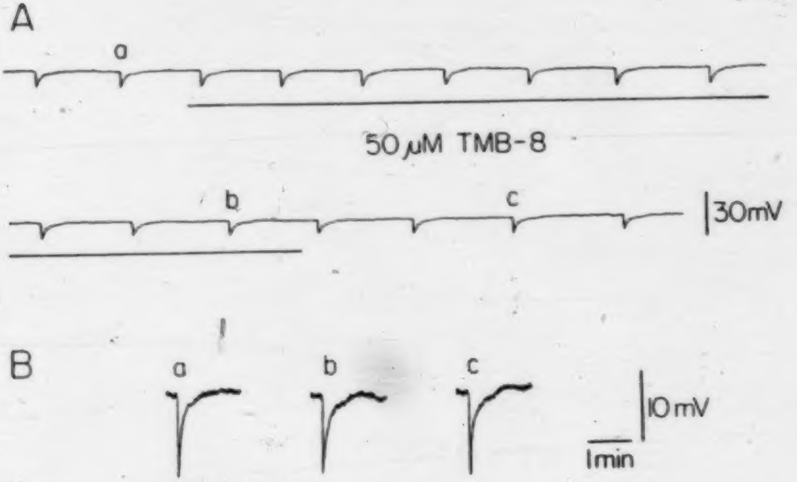


Fig. 57. Lack of effect of TMB-8 on sCaff$_H$ recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. A. Low gain continuous record of membrane potential of a B cell in the presence of 5 mM caffeine and 50 μM TMB-8. Caffeine was superfused continuously whereas 50 μM TMB-8 was applied for the period indicated by the horizontal bars. B. High gain records of sCaff$_H$'s before, Ba; during, Bb; and after, 50 μM TMB-8, Bc. Lower case letters indicate corresponding responses in A. Traces were obtained from a rectilinear pen recorder.

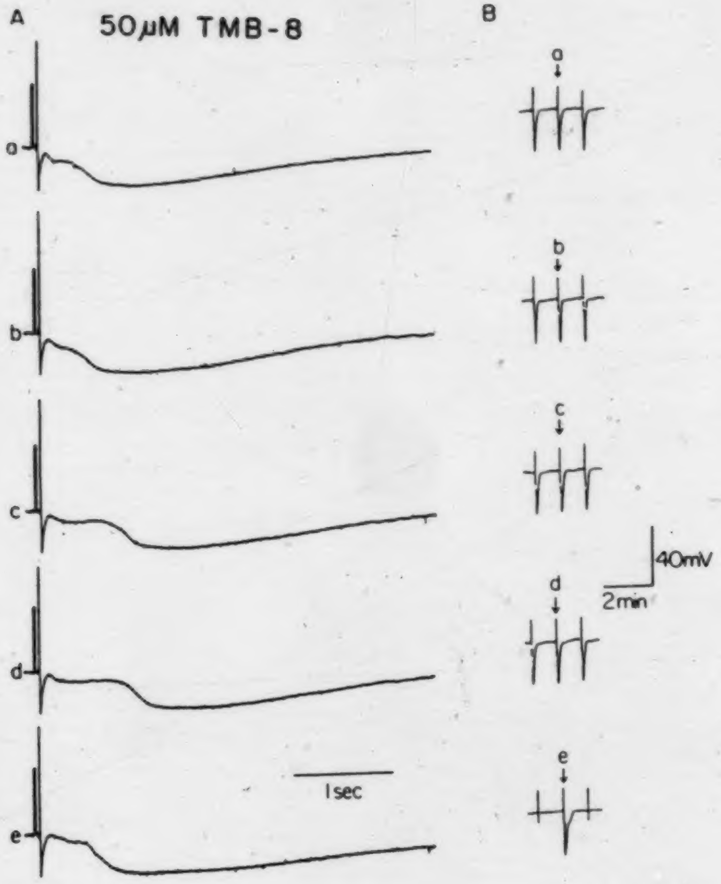


Fig. 58. Effect of TMB-8 on the $Caff_H$ induced by an antidromically evoked action potential recorded intracellularly from a Rana catesbeiana sympathetic ganglion B cell. Aa, control $eCaff_H$; Ab, $eCaff_H$ following 10 min in 50 μM TMB-8; Ac, $eCaff_H$ 3.5 min after TMB-8; Ad, $eCaff_H$ 5 min after TMB-8; Ad, $eCaff_H$ 5 min after TMB-8; Ae, $eCaff_H$ 23 min after TMB-8. Traces in A were obtained from a digital storage oscilloscope. Voltage calibrations preceeding action potentials are 50 mV, 20 msec. B. Same traces (arrow) as in A obtained from a rectilinear pen recorder.

the threshold for intracellular Ca^{2+} release without affecting the amount of Ca^{2+} liberated.

6. Summary - calcium activated potassium conductance

The results from sections 4, 5 and 6 are summarized in Table 9. Only quinidine affected all three responses: the Ad_H, $sCaff_H$ and $eCaff_H$, in an inhibitory fashion. TEA and apamin could be grouped together since they both had little effect on the Ad_H while selectively depressing the fast initial component of the $sCaff_H$'s. 4-AP and TMB-8 had somewhat similar effects in that they both depressed the Ad_H but had no effect on the $sCaff_H$. Dantrolene, while having no discernible effect on the Ad_H, has been reported by Kuba (1980) to produce variable inhibitory actions on the $Caff_H$'s On the whole, it would appear that these six agents affected the Ad_H, recorded by the sucrose-gap technique, and the $Caff_H$'s, recorded intracellularly, in substantially different ways. This would suggest that the Ad_H and $Caff_H$'s are not produced by identical ionic mechanisms.

Table 9

Effects of various potassium channel blockers and intracellular Ca^{2+} stabilizing agents on the Ad_H, sCaff and $eCaff_H$

	Ad_H	$sCaff_H$	$eCaff_H$
10 mM TEA	98.3±15.6%(3)[a]	39.6±8.0%(4)	not tested
1mM 4-AP	17.2± 5.4%	no effect(8)	not tested
50μM Quinidine	37.6±10.6%(6)	58.1±2.0%(4)	37.5%(1)
500 nM Apamin	no effect(7)	63.6%(1)	34.8%(1)
60μM Dantrolene	no effect(7)	not tested	not tested
50μM TMB-8	38.9±7.1%(7)	no effect(6)	no effect(2)[b]

a - In 5 other preparations, 10 mM TEA caused the resting sucrose-gap potential to become very unstable, thus obscuring the Ad_H's elicited during the TEA application

b - In 2 cells, the latency of onset of the eCaff was reversibly lengthened but the amplitude of the response was unaffected

CHAPTER IV

DISCUSSION

A. Effects of adrenaline recorded by sucrose-gap and intracellular methods

1. Explanation of the results in terms of differences between sucrose-gap and intracellular methods

An aspect of this study which demands immediate and thorough consideration is the striking contrast between the hyperpolarizing effect of adrenaline on the whole sympathetic ganglion recorded by the sucrose-gap method and the complete lack of effect of adrenaline on single ganglion B and C cells recorded by the intracellular method (see Section A of the Results). Concentration-dependent hyperpolarizations to superfused adrenaline (Ad_H) could be consistently obtained in sympathetic ganglia of both Rana catesbeiana and Rana pipiens. Occasionally, (2 out of 10 preparations, Rafuse and Smith, 1982) depolarizations to adrenaline (Ad_D's) were recorded in Rana catesbeiana but never in Rana pipiens. All of the intracellular experiments were performed on Rana catesbeiana but neither a hyperpolarization nor a depolarization could be convincingly recorded in response to superfused adrenaline. There may be several possible reasons for this.

Section E of the Introduction gives detailed descriptions of the sucrose-gap and intracellular recording techniques. The point is emphasized that, even when the same preparation is used in both cases, the two methods record from different parts of the neuronal membrane. In the bullfrog sympathetic ganglion, the sucrose-gap method records the average compound membrane potential of a population of axon-hillock and axonal membranes, while the intracellular method records the membrane potential of a single cell body. It is explained that non-propogating signals (eg. drug responses) originating in the cell bodies of the ganglion would electrotonically spread down the axons to be detected by sucrose-gap recording but that the amplitude of the signal would be attentuated according to the distance and cable properties of the axons. Alternatively, if a drug response originates in the axon or axon-hillock region of the neurone, it would spread into the cell body as well as down the axon. Such a response would be detected by sucrose-gap recording more easily than by intracellular recording because the cable properties for electronic spread are more favourable for a cylindrical axon than a spherical or oblong cell body (Jack, et al., 1975). If the α_2-adrenoceptors which mediate the Ad_H (recorded by means of the sucrose-gap technique) reside on the axon or even the axon-hillock, then the electrotonic invasion of the cell body may not be sufficient for the intracellular recording electrode to detect anything.

Koketsu and Nakamura (1976) were also unable to record an Ad_H in either B or C cells of bullfrog sympathetic ganglia using conventional intracellular methods. These authors proposed that the Ad_H originated at the axon-hillock membrane for the following reasons. 1) The Ad_H could easily be recorded by the sucrose-gap technique which records from this membrane as well as the axon. 2)

Concentrations of adrenaline up to 300 μM had no effect on isolated postganglionic nerve trunks, suggesting that the receptors were not on the axonal membrane. 3) Presynaptically evoked B spikes were examined before and during the Ad_H. It was found that while the overall voltage deflection from the peak of the action potential to the bottom the afterhyperpolarization did not change, the ratio of the spike amplitude to afterhyperpolarization amplitude increased during the Ad_H. This was taken to indicate that the Ad_H occurred at least on the B cell membrane.

Koketsu and Nakamura (1976) may have been correct about the Ad_H not originating in the cell body of B and C cells, and the response may very well originate in the axon-hillock membrane, but they really have not ruled out the initial segment of the axon (IAS) itself. First of all, regardless of how the frog ganglion is positioned in the sucrose-gap chamber, adrenaline applied to the ganglion will come in contact with short segments of axons (IAS) of the more rostrally situated cells. There may not be any receptors for adrenaline on axonal membranes remote from the ganglion, as Koketsu and Nakamura have demonstrated using a piece of the postganglionic nerve trunk, but this membrane may be very different from the membrane of the IAS. Secondly, the third experiment described may not have been correctly interpreted in light of more recent data. Minota and Koketsu (1977) have shown in bullfrog sympathetic ganglion cells and McAfee and his colleagues have shown in mammalian ganglia (Horn and McAfee, 1980;

McAfee, 1981) that α-adrenergic agonists reduce the amplitude of the action potential afterhyperpolarization (AP_{AH}) by antagonizing a voltage-dependent Ca^{2+} influx which activates a gK involved in the AP_{AH}. This action of adrenaline might at least partially explain the effect of adrenaline on the ratio of the amplitude of the spike to the amplitude of the AP_{AH}. It should also be remembered that action potentials recorded by the sucrose-gap method are also detected (as is the Ad_H) in the axon-hillock region and IAS of the cells more rostrally positioned in the ganglion.

Even if the α_2-adrenoceptors and K^+ channels which mediate the Ad_H reside on the cell body membrane, it is possible that the membrane disruption produced by the penetration of an intracellular electrode may somehow affect the ion channels which generate the Ad_H. It has recently been shown, for example, that the late Ca^{2+}-activated K^+ current underlying the AP_{AH} (I_{AHP}) described by Pennefather, et al. (1985) can only be recorded when penetration injury is minimal. Also prior to the impressive demonstration by Horn and Dodd (1981), of slow i.p.s.p.'s approaching 40 mV in amplitude, most investigations had reported some difficulty in recording slow i.p.s.p.'s from curarized C cells (for review see Kuba and Koketsu, 1978). In light of the technical improvements which have been made allowing a large slow i.p.s.p. to be recorded intracellularly (Horn and Dodd, 1981), it may eventually be possible to record an Ad_H intracellularly with even further technical refinements.

2. Effects of adrenaline on B and C neurones

Another fundamental difference between the sucrose-gap and intracellular recording techniques is that the former records average membrane potential changes of a population of ganglion cells while the latter directly records the membrane potential of a single neurone. It is widely accepted that two general cell types exist in amphibian sympathetic ganglia: B and C cells (see Introduction). It is conceivable, that only one of these cell types responds to adrenaline.

Of the 62 ganglion cells examined for responsiveness to adrenaline, only 8 were C cells; the remaining 54 were B cells (See Section A of the Results). As was previously mentioned, neither cell type appeared to respond in a reproducible manner to adrenaline. It may be significant to the discussion on the adrenergic hypothesis of the slow i.p.s.p. that C cells were equally as unresponsive to adrenaline as were B cells (see section E of Discussion). Nevertheless a slow i.p.s.p. can be recorded intracellularly from C cells (Weight, 1983; Dodd and Horn, 1983b).

Most sucrose-gap recordings of the Ad_H were made from the IXth and Xth ganglion. Occasionally, the VIIIth ganglion in *Rana pipiens* was used when neither the IXth or Xth rami communicans were long enough to span the sucrose-gap. Although, no data was presented on this ganglion, no particular difficulty were experienced obtaining Ad_H's of amplitudes comparable to those in the IXth and Xth ganglia. This is interesting because it is not known whether this ganglion contains any C cells (see Fig. 1 from Skok, 1973). If this is the case, it would suggest that the Ad_H is not exclusively generated in C cells as is believed to be true for the slow i.p.s.p.

B. Adrenaline induced hyperpolarization and electrogenic NaK-pump stimulation

1. General comments

Koketsu and Nakamura (1976) proposed that the Ad_H in bullfrog sympathetic ganglia was generated by activation of the electrogenic NaK-pump. Phillis and Wu (1981) extended this hypothesis to explain their own observations, and the observations of others, concerning catecholamine induced inhibitory responses in excitable membranes in general. Three pieces of evidence are persistently used in support of this hypothesis (Phillis and Wu, 1981). 1) The majority of investigators who had succeeded in recording a catecholamine induced hyperpolarization in vertebrate neurones had not succeeded in measuring an increase in membrane conductance during the response 2) Catecholamine induced inhibitory responses (including hyperpolarizations) were shown to be selectively inhibited by the cardiac glycoside, ouabain. 3) Catecholamines could enhance ouabain-sensitive ATPase activity in isolated neural membrane homogenates.

With respect to studies on mammalian sympathetic ganglia, it must be conceded that a convincing change in membrane conductance has not been measured in response to catecholamines. In amphibian ganglia, not even a convincing membrane potential change (depolarization or hyperpolarization) has been recorded intracellularly to adrenaline (see section A2 of the Results). It should be noted however that since the review by Phillis and Wu (1981) appeared, clear conductance increases to potassium have been reported following α_2-adrenoceptor activation in locus coeruleus (Aghajanian and Vander Maelen, 1982; Egan, et al., 1983), substantia gelatinosa (North and Yoshimura, 1984), myenteric neurones (North and Suprenant, 1985) and parasympathetic ganglia (Akasu, et al., 1985). The failures to demonstrate responses and conductance changes to catecholamines in sympathetic ganglia may be explained by one or more of the suggestions made in Section A of the Discussion.

The second point by Phillis and Wu (1981) claims that catecholamine induced inhibitory responses are selectively inhibited by cardiac glycosides. This has not been persuasively demonstrated in sympathetic ganglia. In amphibian sympathetic ganglia, Koketsu and Nakamura (1976) reported that 1 μM ouabain (applied for more than 60 min) had very little effect on the Ad_H. Depression of the Ad_H to 50% of control was only observed following at least 60 minutes exposure to 10 μM ouabain. Smith (1984) showed that 10 μM ouabain inhibited the ACh_{AH} (ie. electrogenic NaK-pumping) to 25% of control in 40 minutes without having a statistically significant effect on the Ad_H. Fig. 36 in Section D3 of the Results gives an example of an ACh_{AH} which was completely eliminated following 47 min in 10 μM ouabain. The other electrogenic NaK-pump paradigm studied, the K_H, was reversed (ie. NaK-pump blocked) in Fig. 38A following 69 min exposure to 1 μM ouabain. On the basis of these data it would seem reasonable to suspect that the effect of ouabain on the Ad_H is secondary to NaK-pump inhibition.

The third point contends that catecholamines could enhance NaK-ATPase activity in broken membrane preparations (Phillis and Wu, 1981). This may not have any relevance to catecholamine actions in intact tissues. The evidence presented in Section D5 of the Introduction favours a non-receptor mechanism whereby catecholamines remove or inactivate soluble inhibitory factors (possibly vanadate, see next section of the Discussion); to produce apparent "stimulation" of the enzyme. This mechanism does not involve a particular adrenoceptor. The Ad_H, however, seems to be mediated by an α_2-adrenoceptor (see Section B of the Results).

Differential effects of the divalent cation, Ba^{2+}, were noted on the Ad_H and ACh_{AH} in Section E1 of the Results. While the Ad_H was reduced to 11% of control by 2 mM Ba^{2+}, the ACh_{AH} to ACh_D ratio was enhanced to 192% of control. This is a clear demonstration that the Ad_H is not generated by the same mechanism as is the ACh_{AH} (ie. electrogenic NaK-pumping). In fact, these results are easily explained by the K^+ channel blocking action of Ba^{2+} (see Section E1 of the Results).

In conclusion, it would appear that there are a number of serious inconsistencies concerning the hypothesis of Koketsu and Nakamura

(1976) which explains the Ad_H in terms of electrogenic NaK-pumping. The inability to record the Ad_H intracellularly makes an accurate characterization of the ionic mechanism of the response very difficult. Consequently, the investigator has been left with the sucrose-gap technique to compare the sensitivities of the Ad_H and electrogenic NaK-pump responses, such as the ACh_{AH} and K_H, to various pharmacological agents. In the following section of the Discussion, the NaK-ATPase inhibitor, vanadate, is evaluated as a useful tool for studying putative NaK-pump generated responses.

2. Vanadate as a tool for studying the electrogenic NaK-pump

Vanadate had been shown to inhibit NaK-ATPase activity isolated from a variety of sources with a potency comparable to or even greater than ouabain (for review see Nechay, 1984). Further interest in this NaK-pump inhibitor was elicited by suggestions that endogenous vanadate and catecholamines might interact to play a physiological role in the regulation of active ion transport (Cantley, et al., 1977; Beauge and Glynn, 1977). It was proposed that catecholamines might stimulate NaK-ATPase by reversing a tonic inhibiton by vanadate (Cantley, et al., 1978; Hudgins and Bond, 1979; Adam-Vizi, 1980, also see Introduction). This possibility was also addressed by the experiments described in Section D of the Results.

a) Comparison with ouabain

The most important findings in Section D of the Results are summarized in Table 10. In a previous study, Smith (1984) compared

Table 10

Comparison of the effects of Na_3VO_4 and ouabain on the Ad_H, K_H and ACh_{AH}

	Na_3VO_4	Ouabain
Ad_H	↓ rapidly	↓ slowly
K_H	no effect	↓
ACh_{AH}	no effect	↓

the effects of ouabain on the Ad_H and ACh_{AH}. It was found that the ACh_{AH} was much more sensitive to ouabain than was the Ad_H. Employing a similar approach with vanadate in this study, it was found that 1 mM Na_3VO_4 produced no effect whatsoever on the ACh_{AH} but the Ad_H was rapidly reduced to 31.6% of control (n=13). This high concentration of Na_3VO_4 also had little effect on the K_H. It is interesting to note that brief applications of 400 μM $NaVO_3$ also had no effect on the K_H measured in mouse skeletal muscle (Dlouha, et al., 1981). The lack of effect of vanadate on the two NaK-pump paradigms was interesting in light of the fact that ouabain and vanadate both inhibited the isolated NaK-ATPase from bullfrog spinal cord and brain at concentrations several orders of magnitude less than the 1 mM superfused over the NaK-pump *in situ*. Since it is known that ouabain acts at an extracellular site on the enzyme while vanadate acts at a cytoplasmic site (see *Introduction* and Fig. 4), it was reasonably concluded that vanadate was unable to gain access to its site of action when applied externally (by superfusion) to intact cells. Therefore, it was suspected that vanadate produced its inhibitory effect on the Ad_H by an extracellular mechanism.

b) *Vanadate uptake*

In order to thoroughly rule out the possibility that vanadate might be affecting the Ad_H by an intracellular mechanism, the anion exchange blocker DNDS and the Cl^- channel blocker anthracene-9-carboxylic acid (A9CA) were examined for any effects (possible reversal) on the vanadate induced inhibition of the Ad_H. Neither compound significantly altered the characteristic inhibition of the Ad_H by vanadate (see Section D4 of the Results).

c) Chemical inactivation of adrenaline by vanadate

It was a serious concern that the apparent inhibition of the Ad_H in the presence of vanadate might simply reflect chemical oxidation of the catecholamine by Na_3VO_4 in the Ringer's solution bottle; (see Section D2 of the Results). The oxidation-reduction reaction proposed to occur between vanadate and the catechol moiety is shown in Fig. 4 of the Introduction. Control experiments, including the one illustrated in Fig. 35 made it clear, however, that an Ad_H could occur, albeit slightly depressed, even after the adrenaline had been mixed with Na_3VO_4 (in a stoichiometric ratio of 1 adrenaline : 1000 Na_3VO_4) for 2 full hours. Cantley and his colleagues (1978a) have reported that at high catechol to vanadate ratios, a blue colour characteristic of tetravalent vanadyl appears instantaneously upon mixing. Such a blue colour was never observed in the present experiments. Initially, it was suspected that the presence of the antioxidant $NaHSO_3$ (1/10th concentration of adrenaline) might be protecting the oxidation of adrenaline to a quinone compound, but the same results were obtained when $NaHSO_3$ was omitted from the Ringer's solution. In a verbal discussion with Cantley, he suggested that the extreme ratio of 1 catechol : 1000 Na_3VO_4 might not permit the reaction to occur as he has previously reported. Another possible explanation of the results might be that the reaction does occur and the adrenaline is fully oxidized but that the resulting quinone compounds are as effective as catecholamines in inducing a hyperpolarization in Rana pipiens sympathetic ganglion cells.

d) Magnesium dependence of vanadate inhibition

It has been widely reported that Mg^{2+} or other divalent cations such as Ca^{2+} or Mn^{2+} are necessary for the binding of vanadate to the NaK-ATPase and inhibition of the enzyme activity (for review see Nechay, 1984). Mg^{2+} promotes the $E_1 \rightarrow E_2$ conformational change of the enzyme and it is the E_2 state in which vanadate "traps" the enzyme (Smith, et al., 1980). The optimal $[Mg^{2+}]$ for vanadate inhibition of the dog kidney NaK-ATPase was approximately 25-28 mM (Cantley, et al., 1977). In this study the K_i for vanadate inhibition changed from 250 nM at physiological free $[Mg^{2+}]$ of 600 nM to 40 nM at 25 mM Mg^{2+}. In red cell ghosts, the change in K_i was from 100 nM (at 4 mM Mg^{2+}) to 40 nM (at 25 mM Mg^{2+}) (Cantley, et al., 1978). The experiments in Section D1 of the Results indicate smaller changes in the IC_{50} values in both the bovine brain (from 690 nM at 2 mM Mg^{2+} to 420 nM at 22 mM Mg^{2+}) and frog CNS NaK-ATPases (from 3.0 μM at 2 mM Mg^{2+} to 2.5 μM at 28 mM Mg^{2+}). It is curious that these enzymes were relatively insensitive to changes in $[Mg^{2+}]$, particularly when the basal level of Mg^{2+} in the incubation mixture was only 2 mM and 200 μM EDTA was also present. The presence of the EDTA would make it unlikely that trace amounts of contaminating divalent cations such as Ca^{2+} would measureably facilitate vanadate inhibition of the NaK-ATPase. It would appear on the basis of these data that vanadate inhibition of the bovine brain and bullfrog CNS enzymes were not particularly dependent on the presence of divalent cations such as Mg^{2+}.

e) How does vanadate antagonize the Ad_H?

It would seem fairly certain that vanadate does not inhibit the electrogenic NaK-pump in the intact *Rana pipiens* sympathetic ganglion even though NaK-ATPase isolated from *Rana catesbeiana* CNS tissue is inhibited in a concentration-dependent manner. The simplest explanation of these observations is that vanadate is unable to cross the neural membrane in sufficient quantities to inhibit the NaK-pump from the cytoplasmic surface. The question remains; how does vanadate produce its inhibitory action on the Ad_H?

It should be remembered that vanadate is only a specific inhibitor of the NaK-ATPase at concentrations less than 1 μM. Due to its structural resemblance to phosphate, vanadate has been demonstrated to inhibit a large number of phosphohydrolases (for a list of enzymes and apparent inhibition or dissociation constants see Macara, 1980). One enzyme which is not inhibited, but is instead stimulated by vanadate is adenylate cyclase. Since the Ad_H appears to be mediated by an α_2-adrenoceptor (see Section B of the Results), and since α_2-adrenoceptor stimulation is usually linked to a decrease in cyclic AMP levels (for reviews See Exton, 1982; Limbird, 1983), it is tempting to speculate that vanadate may inhibit the Ad_H by stimulating adenylate cyclase activity. Andrade and Aghajanian (1984) have made a preliminary report that both clonidine and opiate induced hyperpolarizations (two responses associated with decreases in cyclic AMP levels) in locus coeruleus could be antagonized by dibutyryl and 8-bromo cyclic AMP. Also, Smith and Zidichouski have observed (unpublished data) that the Ad_H in Rana catesbeiana sympathetic ganglia was almost completely eliminated by the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX).

Earlier in the Discussion, it was stated that it was unlikely that orthovanadate penetrated the intact neuronal membrane since it had no effect on the ACh_{AH} or K_H (NaK-pump paradigms). It might also be expected that orthovanadate should only stimulate adenylate cyclase activity by an intracellular mechanism.

However, the exact mechanism by which vanadate stimulates adenylate cyclase activity has not been fully elucidated. It has been tentatively proposed to act on the guanine nucleotide regulatory protein similarly to, but not exactly the same as, F^- (Krawietz, et al., 1982). This mechanism might also be expected to require uptake of vanadate into the cytoplasmic space, but investigations on intact cardiac muscle have shown that cyclic AMP increases could be measured in response to vanadate concentrations which also produced positive inotropy (Grupp, et al., 1979; Hackbarth, et al., 1980).

3. Evaluation of the "vanadate reversal" hypothesis

Several groups have reported that millimolar concentrations of catecholamines could reverse vanadate inhibition of the NaK-ATPase (Cantley, et al., 1977: Quist and Hokin, 1978; Hudgins and Bond, 1979; Wu and Phillis, 1979c). On the basis of this observation and the knowledge that pentavalent vanadate is reduced by catecholamines to inactive tetravalent vanadyl (Cantley, et al., 1978a), it was suggested that the commonly observed enhancement of NaK-ATPase activity by catecholamines might not be due to direct enzyme stimulation but rather by chemical inactivation of endogenous vanadate and thus removal of its inhibitory influence (Cantley, 1978a; Hudgins and Bond, 1979).

It is possible that this mechanism explains catecholamine enhancement of isolated NaK-ATPase activity, but it is very unlikely that this mechanism occurs at the electrogenic NaK-pump _in situ_ to explain the vanadate reduction of the Ad_H.

Several of the results presented in this thesis bear this out. 1) It has already been mentioned in earlier sections of the _Discussion_ that vanadate does not appear to be taken up by sympathetic ganglion cells. This is an absolute requirement for this mechanism. It is interesting to note that even though vanadate appears to be readily taken up by red blood cells (by an anion exhange mechanism which is blocked by DNDS), 50% inhibition of $^{86}Rb^+$ uptake requires approximately 1000 x's more vanadate than does inhbition of NaK-ATPase activity from red cell ghosts (Cantley, et al., 1978b). This lack of sensitivity of the intact cells to vanadate probably reflects chemical inactivation (reduction) by high levels of intracellular glutathione (Macara, et al., 1980). 2) Equally as important, to vanadate uptake, is catecholamine uptake. In Section A1 of the Results, the catecholamine uptake blocker, desmethylimipramine (DMI), was shown to potentiate the Ad_H by shifting the concentration-effect curve to adrenaline, in a parallel fashion, to the left. Presumably, this potentiation by DMI reflects blockade of adrenaline uptake. The "vanadate reversal" hypothesis would predict just the opposite effect; blocking catecholamine uptake should reduce catecholamine reversal of vanadate inhibition of the NaK-pump. 3) It seems quite clear that the Ad_H is mediated by a pharmacologically distinct adrenoceptor of the α_2-subtype (see Section B of the Results). The vanadate reversal hypothesis does not implicate an adrenoceptor. Furthermore, adrenoceptors (including α_2-adrenoceptors) are generally accepted to reside on the extracellular surface of the membrane, not the cytoplasmic surface (Bowman and Rand, 1980). 4) The final and most fundamental criticism against this hypothesis providing a mechanism for the Ad_H, is simply that it is unlikely that the Ad_H involves electrogenic NaK-pumping at all (see Section D3 of Introduction). Smith (1984) has shown that the Ad_H is more likely to result from an increase in K^+ conductance (see previous section of the Discussion and Section D of the Introduction). The following section of the Discussion will evaluate the membrane events believed to be associated with the Ad_H.

C. Cellular events associated with the adrenaline induced hyperpolarization

1. General Comments

It is a general principle of neuropharmacology that neurotransmitters and drugs produce their membrane effects by interacting with a cell surface receptor and, by a variety of mechanisms, this receptor activation leads to a change in ion movements across the neural membrane (Cooper, et al., 1982). The final step in this process, the transmembrane ion movement determines whether a hyperpolarization or depolarization results.

A fundamental question in pharmacology is whether there is a functional basis for receptor classification. Is a particular receptor subtype always linked to a particular sequence of cellular events and thus a particular physiological response? There are a growing number of reports in the literature which support the contention that receptor types are in fact associated with specific cellular events.

The original differentiation of adrenergic receptors into α- and β-adrenoceptors by Ahlquist (1948) was made according to whether adrenergic drugs constricted or relaxed vascular smooth muscle. At the level of the neural membrane, α_1- and α_2-adrenoceptors can be distinguished on the basis of whether they cause an excitatory (depolarizing) or inhibitory (hyperpolarizing) response (Aghajanian and Rogawski, 1983). Furthermore, it would seem likely that

α_1-adrenoceptor mediated depolarizations are due to a K^+ conductance decrease and α_2-adrenoceptor mediated hyperpolarizations are due to a K^+ conductance increase (Aghajanian and Rogawski, 1983; Akasu, et al., 1985). This functional basis for α-adrenoceptor classification is consistent with the generalized scheme proposed by Berthelsen and Pettinger (1977) that α_1-adrenoceptors mediate "excitatory" cellular responses and α_2-adrenoceptors mediate "inhibitory" cellular responses in neuronal as well as non-neuronal cell types. Data is presented in this thesis which suggests the Ad_H recorded from frog sympathetic ganglia results from an α_2-adrenoceptor mediated increase in K^+ conductance (Section E1 of the Results; see also Smith and Rafuse, 1983; Smith, 1984a, 1984b). In agreement with this finding are the α_2-adrenoceptor mediated increases in K^+ conductance (hyperpolarizations) which have been reported in locus coeruleus (Egan, et al., 1983; Williams, et al., 1985), myenteric ganglia (North and Suprenant, 1985), substantia gelatinosa (North and Yoshimura, 1984) and parasympathetic ganglia (Akasu, et al., 1985).

It would also appear that receptors may be preferentially linked to specific receptor-effector transduction mechanisms and second messengers. As examples, β-adrenoceptors are usually associated with cyclic AMP increases (Nathanson, 1977), α_1-adrenoceptors with increases in cytosolic Ca^{2+} (Exton, 1982) and α_2-adrenoceptors have been linked with decreases in cyclic AMP levels (Exton, 1982; Limbird, 1983). The nicotinic ACh receptor and GABA receptor represent another type of receptor system which does not involve a second messenger since the receptors and ion channels are believed to be directly connected in a macromolecular complex (Kandel and Schwartz, 1981).

Although activation of a specific receptor may produce a particular neural membrane response, it would appear that there is some redundancy of receptor mediated effects in the CNS. Inhibitory catecholamine responses in various CNS membranes may be mediated by either α_2- or β-adrenoceptors depending on the brain area (Szabadi, 1979; Aghajanian and Rogawski, 1983). It is believed that the α_2-adrenoceptor mediated hyperpolarization in the locus coeruleus for example, is due to an increase in gK (Aghajanian and Vander Maelen, 1982; Williams, et al., 1985) but the ionic mechanism of the β-adrenoceptor mediated inhibitory effects has not been established with any certainty. It would be interesting to determine whether the β-adrenoceptor effect occurs as a result of an ionic mechanism similar to or different from that of the α_2-adrenoceptor mechanism (see Section C2 of Introduction for possible alternatives).

2. Receptor classification - Ad_H

a) Antagonist studies

The adrenergic blockers used in these studies provided reasonably unambiguous support for the assertion that a α_2-adrenoceptor mediates the Ad_H (see Section B3 of the Results). Concentrations of the β-blockers, sotalol and propanolol; the DA antagonist, chlorpromazine; and the α_1-blocker, prazosin, which were equimolar to the applied adrenaline failed to produce any appreciable inhibitory effect on the Ad_H. Chlorpromazine occasionally reduced the Ad_H, presumably due to its α-blocking activity (Bowman and Rand, 1980). In contrast to these ineffective antagonists, the α_2-adrenoceptor antagonists yohimbine (IC_{50} = 6.2 nM) and idazoxan (IC_{50} = 590 nM), and the non-selective α_1- and α_2-adrenoceptor blocker, phentolamine (IC_{50} = 530 nM) antagonized the Ad_H. Yohimbine proved to be the most effective antagonist with an IC_{50} even lower than the majority of reports on yohimbine induced release of neurotransmitters (for review see Goldberg and Robertson, 1983). Phentolamine and idazoxan appeared to be much less potent, compared with previous reports of postsynaptic α_2-adrenoceptor blockade in locus coeruleus (K_e: phentolamine, 20 nM; idazoxan, 9 nM; Williams, et al., 1985) and myenteric neurones (IC_{50}: phentolamine, 1.8 nM; idazoxan, 4.5 nM; North and Suprenant, 1985). Phentolamine and idazoxan have also been observed to block presynaptic α_2-adrenoceptors in rat vas deferens (Doxey, et al., 1983) with affinities similar to those found for the postsynaptic α_2-adrenoceptor by North and his colleagues. It is difficult to reconcile the differences in effectiveness of these two α-blockers in antagonizing the Ad_H in frog sympathetic ganglia and the α_2-adrenoceptor effects in mammalian neurones (Doxey, et al., 1983; North and Suprenant, 1985; Williams, et al., 1985). Although the weight of evidence supports the suggestion that the Ad_H is mediated by an α_2-adrenoceptor, this receptor may not be identical to the mammalian α_2-adrenoceptor (cf. also Brown and Caulfield, 1979).

b) Agonist studies

Further evidence in support of the conclusion that the Ad_H was mediated via an α_2-adrenoceptor was that the α_2-adrenoceptor agonist, αMeNA (Starke, et al., 1975) produced a concentration-dependent hyperpolarization ($\alpha MeNA_H$) which (like the Ad_H) was reduced by the α_2-adrenoceptor antagonist, idazoxan (see Fig. 23). Also, the lack of effect of methoxamine makes it unlikely that an α_1-adrenoceptor mediates the Ad_H. Clonidine (CLN) on the other hand, only weakly hyperpolarized the frog ganglion cells (see Section B1 of the Results and Fig. 22A). The efficacy of CLN in hyperpolarizing the rat superior cervical ganglion was also very low in comparison to noradrenaline (Brown and Caulfield, 1979, but cf. Williams, et al., 1985). This might not be surprising in light of the demonstration by Bousquet and his colleagues that αMeNA and CLN produce their centrally mediated hypotensive action through different brain areas and possibly different catecholamine (αMeNA) preferring and imidazoline (CLN) preferring sites (Bousquet, et al., 1984). It has been claimed that imidazoline compounds such as CLN may act as partial agonists on α_2-adrenoceptors (Starke, 1977; Bowman and Rand, 1980; also see the Appendix for acomparison of the chemical structures of Ad, αMeNA and CLN). This might explain the low efficacy of CLN in frog and mammalian sympathetic ganglia. Interestingly, CLN has been reported to be more potent than and just as efficacious as NA in hyperpolarizing locus coeruleus neurones (Williams, et al., 1985), although the rates of onset and offset of the CLN_H were much slower than the NA_H as observed in sympathetic ganglion cells (cf. Brown and Caulfield, 1979). In this tissue, phentolamine reduced both the NA_H and CLN_H yet desmethylimipramine (DMI) potentiated the NA_H but blocked the CLN_H (Williams, et al., 1985). The differential effects of DMI could be interpreted to suggest that NA and CLN act on different receptors or act at different sites on the same α_2-adrenoceptor. The presence or absence of DMI had no effect on the small CLN_H's recorded in Rana pipiens sympathetic ganglia (see Section B2 of Results).

Other agonist data could infer that DA and β-adrenoceptors are not involved in the Ad_H. While DA and INA (β-adrenoceptor agonist) also produced hyperpolarizing responses, they rapidly and irreversibly desensitized whereas the Ad_H remained relatively constant. One interpretation of the lack of cross-desensitization is that DA and INA act on different receptors from the α_2-receptors involved in the Ad_H. This method of cross-desensitization is not a definitive approach for the classification of receptors since it does not rule out the possibility that two different agonists act at different sites on the same receptor. The rapid desensitization of the DA_H and INA_H also precluded the testing of β-adrenergic and DA selective antagonists on Rana pipiens ganglia (see Section B2 of Results). In contrast to frog ganglia, hyperpolarizations to DA and

INA in rat superior cervical ganglion appeared resistant to desensitization (Brown and Caulfield, 1979). These workers showed that DA_H was convincingly antagonized by the α_2-adrenoceptor blocker, yohimbine, but not by DA antagonists. It was concluded in this study that DA hyperpolarized the mammalian ganglion cells via activation of an α_2-adrenoceptor instead of a DA receptor (Dun, et al., 1977). Furthermore, Brown and Caulfield (1979) showed that while the INA_H was unaffected by propranolol ($\leq$ 10 μM), it was substantially diminished in 1 μM phentolamine. A possibility which cannot as yet be ruled out is that DA, INA and CLN may be acting as partial agonists on α_2-adrenoceptors in frog and mammalian ganglia. It would be an interesting experiment to examine whether the Ad_H is affected by prolonged exposure of the frog ganglion to high concentrations of DA, INA or CLN. Following desensitization of their agonist activity, antagonism of the Ad_H might be expected if they act as partial agonists on the α_2-adrenoceptor.

3. α_2-adrenoceptor mechanisms

One of the most intriguing possibilities which emerges from the proposal that the Ad_H is mediated by an α_2-adrenoceptor is that this response might involve the same cellular events which occur upon presynaptic α_2-adrenoceptor activation and inhibition of neurotransmitter release. It may be reasonable to suspect that these two effects (postsynaptic Ad_H and presynaptic inhibition of transmitter release) are produced by identical mechanisms since they are mediated by identical receptors (see Section C1 of Discussion on functional classification of receptors). If this prospect is seriously considered, insight into the cellular mechanism of presynaptic α_2-adrenoceptor mediated inhibition may be more readily gained by studying the more readily accessible Ad_H. The Ad_H may be conveniently recorded by the sucrose-gap technique whereas direct recording from vertebrate nerve terminals is not yet technically feasible.

It is reasoned that hyperpolarization of nerve terminals would reduce neurotransmitter release (North and Williams, 1983a; Williams, et al., 1985). This could occur by simply lowering the excitablilty of the terminals by moving the membrane potential away from the threshold for action potential generation and blocking the invasion of a propagating impulse into a nerve varicosity. Secondly, it has been proposed that hyperpolarizations due to an increase in K^+ conductance may indirectly block calcium entry (North and Williams, 1983a; 1983b; Williams and North, 1985). In a manner analogous to the proposed mechanism for opiate blockade of transmitter release (North and Williams, 1983a; 1983b), an α_2-adrenoceptor activated increase in K^+ conductance may shorten the duration of the action potential and thus the time for voltage-gated Ca^{2+} to enter the nerve terminal.

While low concentrations of catecholamines hyperpolarize vertebrate neurones, higher doses (> 10 μM) have been reported to directly block a voltage-sensitive Ca^{2+} conductance (Minota and

Koketsu, 1977; Horn and McAfee, 1980; Dunlap and Fischbach, 1981; McAfee, et al., 1981; Galvan and Adams, 1982). McAfee and his colleagues have specifically suggested that an α_2-adrenoceptor mediates this effect in mammalian sympathetic ganglion cells (McAfee, et al., 1981). Theoretically, this mechanism could also provide a means for α_2-adrenoceptor inhibition of neurotransmitter release. However, the assertion that this effect is mediated by an α_2-adrenoceptor has been disputed by Williams and North (1985). Based on studies in locus coeruleus, it is argued by these investigators that an α_2-adrenoceptor is not involved in the direct Ca^{2+} channel effect because the effect was 1) insensitive to yohimbine, 2) was not produced by clonidine and 3) was produced by concentrations of noradrenaline much higher than necessary to activate the hyperpolarizing α_2-adrenoceptor.

In an interesting scheme which relates the two ionic events postulated to result from α_2-adrenoceptor activation (ie. decrease in voltage sensitive Ca^{2+} conductance and increase in K^+ conductance), it has been suggested that they can both result from an increase in intracellular Ca^{2+} (Horn and McAfee, 1980; Jenkinson, 1983). It has been shown in invertebrate neurones that increases of intracellular Ca^{2+} concentration decrease Ca^{2+} influx (Kostyuk and Krishtal, 1976; Eckert and Ewald, 1982). Also, Ca^{2+} activated gK's have been reported in a wide variety of neurones including amphibian (Adams, et al., 1982; MacDermott and Weight, 1982; Brown, et al., 1983; Pennefather, et al., 1985) and mammalian sympathetic ganglia (Horn and McAfee, 1980; Belluzzi, et al., 1985). A tempting synthesis of many of the reports discussed in this section would have low doses of α_2-agonists cause a membrane hyperpolarization due to a Ca^{2+} activated K^+ conductance and higher doses cause a further increase in intracellular Ca^{2+} levels sufficient to block the voltage sensitive influx of Ca^{2+}. A good deal of evidence remains to be obtained to support or refute this speculative proposal. In particular, it must be confirmed whether the α_2-adrenoceptor activated K^+ conductance (Ad_H in frog sympathetic ganglion cells) is in fact dependent on intracellular Ca^{2+} release. However, the data obtained in Section E of the Results and discussed in the following section of the Discussion argue against this possibilty.

4. Is Ca^{2+} involved in the adrenaline induced hyperpolarization?

Although the Ad_H recorded from *Rana pipiens* sympathetic ganglia was unaffected by extracellularly applied 10 mM Mn^{2+} or 100 μM Cd^{2+}, prolonged exposure to a Ca^{2+}-free Ringer's solution caused a progressive decline in the response (see Fig. 43). It should be noted however that Ca^{2+}-free Ringer's solution was not tested against another response other than the Ad_H to examine its specificity of action. In another study (Smith, unpublished observations), it was found that Ca^{2+}-free Ringer's solution antagonized both the ACh_D and the ACH_{AH}. This might suggest that extracellular Ca^{2+} is important for the overall maintenance of the cell membrane and that its absence could have deleterious effects on neurotransmitter and drug responses in general. In any event, since a progressive inhibitory action of Ca^{2+}-free Ringer had previously been observed on the $sCaff_H$ recorded intracellularly in Rana catesbeiana ganglia (Kuba and Nishi, 1976), it can be speculated that the Ad_H might also result from an increase in gK activated by intracellularly released Ca^{2+}. This mechanism had previously been proposed for α_1-adrenoceptor mediated hyperpolarizations in smooth muscle (Exton, 1981; 1982) and α-adrenoceptor mediated hyperpolarizations in other non-neuronal cell types (see Section C3b of Introduction). Furthermore, it has been speculated that hyperpolarizing catecholamine responses in other vertebrate neurones might involve activation of a gK by intracellular Ca^{2+} (Horn and McAfee, 1980; Morita and North, 1981; Aghajanian and Vander Maelen, 1982; Jenkinson, 1983; Akasu, et al., 1985).

To test the hypothesis that the Ad_H results from an increase in gK dependent upon intracellular Ca^{2+} release, the Ad_H was compared to the intracellularly recorded $Caff_H$ (spontaneous, $sCaff_H$; and evoked, $eCaff_H$). These caffeine induced hyperpolarizations had previously been established to occur as a result of an intracellular Ca^{2+} linked gK increase (Kuba and Nishi, 1976; Kuba, 1980; see also Section E3a of the Results). The actions of the various agents on the Ad_H and $Caff_H$'s were compared (see Sections E4 and E5 of the Results). The summary presented in Table 9 quite clearly shows that the Ad_H and $Caff_H$'s were affected in dissimilar manners suggesting that the ionic mechanisms for the two responses are different. Comments on the particular effects of each agent are made in the following section of the Discussion.

4. Actions of potassium channel blockers and intracellular calcium stabilizing agents on the Ad_H and $Caff_H$'s.

a) Tetraethylammonium

The effects of 10 mM TEA were examined on the Ad_H and the $sCaff_H$ (see Section E4a of the Results). Variable results were obtained on the Ad_H. Only 3 of the 8 sucrose-gap preparations were stable enough following TEA superfusion to assess its action on the Ad_H. Potentiation and reduction of the Ad_H were observed between preparations and between Ad applications in the same preparation, but the net effect at 30 min exposure was no significant change in Ad_H amplitude. The curious destabilizing effect observed in the other 5 preparations is difficult to explain since this was not observed when recording intracellularly from single cells. Individual B cells typically depolarized 20-40 mV when 10 mM TEA was applied. Although the cell input resistance was not checked before and after TEA application, it would be reasonable to suspect that this large depolarization was due to blockade of resting gK. It is conceivable that the delayed rectifier and Ca^{2+}-activated gK, two voltage-dependent K^+ currents blocked by TEA (Adams, et al., 1982), are operational at the recorded resting potential of these cells.

In contrast to the lack of effect on the Ad_H, TEA (10 mM) selectively inhibited the fast initial component of Type I and Type IV $sCaff_H$'s without affecting the slower secondary component(s). It was also noted, that both phases of the $sCaff_H$ could be reduced by injecting steady depolarizing current into the cell. It could be concluded that the fast primary component of the $sCaff_H$ is due to a voltage-dependent gK that is out of its activation range at depolarized potentials, but Koketsu and his colleagues (Koketsu, et al., 1982) have shown that when cells were voltage clamped at -55 mV, 5 mM TEA still selectively inhibited the initial phase of the $sCaff_H$. Regardless of the precise mechanism whereby TEA reduced the fast, initial component of the sCaff, it can be concluded that this Ca^{++} activated K^+ conductance is not the ionic mechanism which generates the Ad_H since the Ad_H was not affected by TEA in a similar manner.

b) 4-aminopyridine

Differences between the ionic mechanisms of the $sCaff_H$ and Ad_H were clearly revealed by the differential actions of 4-AP (see Section E4b of the Results). While 1 mM 4-AP reduced the Ad_H to 17.2 ± 5.4% of control (n=5) no effect of 1 mM 4-AP on either phase of the $sCaff_H$ was observed in 8 cells. It was mentioned in the Results that 1 mM 4-AP alone appeared to have no effect on the resting or action potentials of these amphibian cells and the A-current in bullfrog ganglion cells has been shown to be relatively insensitive to 4-AP (Adams, et al., 1982). In contrast, 4-AP sensitive channels have been described in mammalian sympathetic ganglion cells (Galvan, 1982;

Belluzzi, et al., 1985). It may be significant that catecholamine induced hyperpolarizations have only been measured intracellularly in mammalian sympathetic ganglion cells (Kobayashi and Libet, 1970; Christ and Nishi, 1971; Horn and McAfee, 1980) and not in amphibian cells (Koketsu and Nakamura, 1976; Kuba and Koketsu, 1978; see Sections A of the Results and Discussion).

While 1 mM 4-AP had no direct effect on the resting or action potentials of the bullfrog cells, 1 mM 4-AP plus 5 mM caffeine induced spontaneous firing which appeared to be both pre-and postsynaptically generated. 4-AP has been shown to increase spontaneous ACh release at the avian neuromuscular junction (Bowman, et al., 1977) and mammalian sympathetic ganglion (Goto and Watanabe, 1981; Simmons and Dun, 1984). This action may explain the action potentials originating presynaptically, but the postsynaptic action potentials are more difficult to explain in terms of previously described effects of 4-AP.

c) Quinidine

Quinidine was the only agent tested against the Ad_H, $sCaff_H$ and $eCaff_H$ which blocked all three responses (see Section E4c of the Results). Although quinidine was used as a tool to study the involvement of a Ca^{2+} activated gK in the Ad_H and $sCaff_H$, it has other pharmacological properties which make it a less than ideal agent for this purpose. It is particularly important to this study that quinidine is also an α-adrenergic blocking agent (Mecca, et al., 1980). This is unfortunate since the Ad_H seems to be mediated by an α_2-adrenoceptor (see Section B of the Results) and quinidine has been shown to displace ^3H-clonidine binding from bovine cortical membranes (K_i = 60 nM; Ciofalo, 1980). Consequently, it cannot be certain whether the inhibitory effect of 50 μM quinidine on the Ad_H is due to an action on a Ca^{2+}-activated gK or an α_2-adrenoceptor.

d) Apamin

In a manner similar to that of TEA, apamin (500 nM) had no significant effect on the Ad_H, but selectively depressed the fast, initial components of the $sCaff_H$ and $eCaff_H$ (see Section E4d of the Results). Unlike TEA, apamin did not cause any change in the resting membrane potential. Although the Sigma Grade apamin prepared from bee venom used in these experiments was approximately 50% pure, it was important to at least qualitatively bioassay the peptide for activity. The contractile response in rabbit jejunum was used to demonstrate that the peptide was active (Muller and Baer, 1979), but the level of activity often seemed to be quite low. As a result, the stated concentration of 500 nM apamin (which assumed 100% purity) was probably much more than twice the actual concentration of active apamin used. Regardless of the final concentration, an inhibitory effect was observed on the $Caff_H$'s but not on the Ad_H. It should be noted that as little as 10 nM apamin significantly antagonized NA induced K^+ efflux from guinea pig hepatocytes (Banks, et al., 1979).

Whereas the utility of many drugs as tools to study pharmacological responses is limited by their lack of specificity (eg. quinidine), apamin may not be an ideal tool for studying Ca^{2+} activated gK's because it may be too specific (Romey, et al., 1984). Apamin does not block all Ca^{2+} activated gK's in all cells. As examples, the Gardos current in red blood cells is unaffected by apamin (Burgess, et al., 1981) and of two Ca^{2+} activated K^{+} conductances in rat skeletal muscle, one is apamin-sensitive and the other TEA-sensitive (Romey and Lazdunski, 1984). Furthermore, a similar situation may exist in bullfrog sympathetic ganglia; the fast outward Ca^{2+} -activated gK (I_{c}) is apamin insensitive whereas the slower I_{AHP} is antagonized (Pennefather, et al., 1985). In the present study apamin blocks only the fast, initial phase and not the slow, secondary phase of the $sCaff_{H}$ and $eCaff_{H}$. Both phases are believed to involve Ca^{2+} activated gK (Koketsu, et al., 1982a). In light of these observations, the lack of effect of apamin on the Ad_{H} cannot be used as evidence against an intracellular Ca^{2+} involvement in this gK. It may be suggested with some caution that if the Ad_{H} is produced by a Ca^{2+} activated gK, then it is not the same as the Ca^{2+} activated gK which generates the fast, initial component of the $sCaff_{H}$ and $eCaff_{H}$.

e) Dantrolene and TMB-8

Both dantrolene and TMB-8 are believed to prevent the release of Ca^{2+} from intracellular storage sites in smooth and skeletal muscles (see Section E5 of the Results). In the report by Kuba (1980) on bullfrog sympathetic ganglion cells, it was shown that dantrolene (6.4-20 μM) either reduced the $sCaff_H$ or lengthened the interval between responses. The second effect was speculated to result from dantrolene elevating the threshold for intracellular Ca^{2+} release without affecting the amount released. This mechanism might also explain the observation of TMB-8 (50 μM) lengthening the latency of onset of the $eCaff_H$ without altering the response amplitude in the present study (see Fig. 58). However, in contrast to the inhibitory action of dantrolene on the $sCaff_H$ (Kuba, 1980), TMB-8 had no effect on the amplitude of any phase of the $sCaff_H$ (6 cells). Dantrolene (60 μM) and TMB-8 (50 μM) also had different effects on the Ad_H in that the former had no effect and the latter caused a reduction in response size to 38.9% of control (7 sucrose-gap preparations for each compound).

The contrasting effects of dantrolene and TMB-8 on the Ad_H may reflect differences in their cellular actions. While both compounds are thought to block intracellular Ca^{2+} release, TMB-8 may further reduce Ca^{2+} entry (Chiou and Malagodi, 1975). This might suggest that drastic alterations in intracellular Ca^{2+} levels may antagonize the cellular events which generate the Ad_H (see also Sections E2 of the Results and C4 of the Discussion). It is curious, however, that if TMB-8 does disrupt intracellular Ca^{2+} levels, why did it not affect the $sCaff_H$? Therefore, while both dantrolene and TMB-8 had dissimilar effects on the Ad_H and $Caff_H$, the experiments with

TMB-8 did not provide any clear insight as to whether there is an intracellular Ca^{2+} involvement in the Ad_H.

6. Conclusions

The assessment of whether the gK which generates the Ad_H is dependent on intracellular Ca^{2+} is complicated by the fact that several different types of Ca^{2+} activated gK's may exist in sympathetic ganglia (Pennefather, et al., 1985). The $sCaff_H$ has been divided into two Ca^{2+}-activated gK's on the basis of selective blockade by 5 mM TEA (fast, initial phase) and 1 μM ACh (slow, secondary phase; Koketsu, et al., 1982a). It is reasonably certain from the present study that the Ad_H does not involve a Ca^{2+}-activated gK similar to the fast, initial component of the $sCaff_H$ since TEA and apamin inhibited the latter and not the former. It is also likely that the Ad_H does not involve a Ca^{2+}-activated gK equivalent to that underlying the slow, secondary component of the $sCaff_H$ since 4-AP and TMB-8 had different ffects on these responses. These data are summarized in Table 11. It should also be noted that while 1 μM ACh reduced this phase of the sCaff (Koketsu, et al., 1982a; Smith, unpublished observations), 1-10 μM ACh had no effect on the Ad_H (Smith and Zidichouski, unpublished observation). The possibility still remains however that the Ad_H is mediated by an intracellular Ca^{2+} mechanism that is different from those involved in the two phases of the $sCaff_H$. In particular, Ad might liberate Ca^{2+} from a different intracellular store than

Table 11

Effects of various agents on the Ad_H and the initial and secondary phases of the $sCaff_H$

	sCaff		
	Ad_H	Initial	Secondary
TEA	↔	↓	↔
Apamin	↔	↓	↔
4-AP	↓	↔	↔
TMB-8	↓	↔	↔
ACh	↔[a]	↔[b]	↓[b]

a From Smith and Zidichouski, unpublished observations

b Koketsu, et al., 1982a

caffeine. This might explain the different effects of TMB-8 (intracellular Ca^{2+} antagonist) on the Ad_H and $sCaff_H$. Due to the apparent diversity of Ca^{2+}-activated gK's, a positive effect of a specific inhibitor on the Ad_H is required to conclude with certainty that such an ionic mechanism underlies this response. For this reason, it would be interesting to examine the effect of charybdotoxin, a protein inhibitor of a high conductance, apamin-insensitive gK activated by intracellular Ca^{2+}, on the Ad_H (Miller, et al., 1985).

It should be clear that while this study does not definitively rule out the possibility that the Ad_H involves intracellular release of Ca^{2+} by a different mechanism, or from different cytoplasmic stores than the $Caff_H$'s, it is unlikely that the gK which generates the Ad_H is activated by intracellularly released Ca^{2+}. If the Ad_H does not involve Ca^{2+} as a second messenger, it may be possible that the α_2-adrenoceptor which mediates the response is directly coupled to the K^+ ionophore (or is the K^+ ionophore) in a similar way as the GABA and nicotinic ACh receptors are linked to their respective ion channels. To further investigate this possibility it would be important to clarify the relationship, if any, between α_2-adrenoceptor activation and cyclic AMP decreases (see Section C3c of the Introduction). It might be expected that if the α_2-adrenoceptor mediated Ad_H is a direct ionophore response, then it should not be affected by adenylate cylase stimulation by forskolin.

D. Evaluation of the hypothesis that a catecholaminergic interneurone mediates the slow i.p.s.p. in frog sympathetic ganglia

1. General comments

Under conditions of nicotinic blockade (eg. in presence of 70 μM d-tubocurarine), adrenaline, methacholine and presynaptic stimulation all produced hyperpolarizing responses in the frog sympathetic ganglion (Ad_H, MCh_H and slow i.p.s.p.; see Section C of the Results). It was first proposed in curarized rabbit superior cevical ganglia that the slow i.p.s.p. might involve an adrenergic interneurone (Eccles and Libet, 1961). According to the disynaptic hypothesis subsequently elaborated by Libet (1970; see also Section A4d of the Introduction and Fig. 3), the Ad_H, MCh_H and slow i.p.s.p. may all be ultimately mediated by the same postganglionic adrenoceptor. This adrenoceptor was originally suggested to be a dibenamine-sensitive α-adrenoceptor. The disynaptic, adrenergic mechanism was assigned to the slow i.p.s.p. recorded in frog ganglia by analogy to the mammalian ganglion (Libet, et al., 1968; Tosaka, et al., 1968). No direct evidence for this hypothesis was given in these papers; in fact dibenamine was even reported to be a poor inhibitor of the frog slow i.p.s.p. (Libet, et al., 1968). The first real evidence was published by Libet and Kobayashi (1974) (see Section A4d of the Introduction).

Weight and Padjen (1973a; 1973b) made the more conservative suggestion that synaptically released ACh might directly activate a hyperpolarizing muscarinic receptor on the postganglionic neurone. The principle evidence in support of this proposal was: 1) adrenergic blockers which antagonized the direct hyperpolarization to catecholamines were ineffective against the slow i.p.s.p. (eg.'s dihydroergotamine, Weight and Smith, 1980; phentolamine, Yavari and Weight, 1981; Dodd and Horn, 1983), 2) low Ca^{2+} and/or Ca^{2+} antagonists which blocked synaptic transmission (slow i.p.s.p.) had no effect on the direct hyperpolarization to muscarinic agonists (Weight and Padjen, 1973b; Weight and Smith, 1980; Horn and Dodd, 1981).

The experiments performed in Section C of the Results further evaluated the applicability of these opposing hypotheses by employing more appropriate pharmacological agents.

2. Effects of desmethylimipramine on the Ad_H, MCh_H and slow i.p.s.p.

The mechanism for inactivation of catecholamines in frog sympathetic ganglia has not been firmly established. There are suggestions in mammalian ganglia (Hanbauer, et al., 1972; Adler-Graschinsky, et al., 1984) that a DMI-sensitive uptake system may be in effect. This system may also be operational in frog ganglia since 500 nM DMI was observed to cause a parallel shift to the left of the log concentration-effect curve to adrenaline (see Section A1b of the Results and Fig. 19). It might therefore be expected that the MCh_H and slow i.p.s.p. would also be potentiated by 500 nM DMI should they involve catecholaminergic transmission. The results of these experiments were straight forward and are illustrated in Fig. 28. While DMI routinely potentiated the Ad_H, it had no effect whatsoever on the amplitudes of the MCh_H and slow i.p.s.p. The results of these experiments would clearly not support the hypothesis of Libet that a disynaptic, adrenergic pathway generates the slow i.p.s.p. in frog sympathetic ganglia.

3. Effects of yohimbine on the Ad_H, MCh_H and slow i.p.s.p.

It was concluded from the studies presented in Section B of the Results that the Ad_H in the *Rana pipiens* sympathetic ganglion was mediated by an α_2-adrenoceptor (see also Section C2 of the Discussion). Yohimbine most effectively antagonized the Ad_H with an IC_{50} of 6.2 nM. It was for this reason that yohimbine was chosen as the adrenergic antagonist to study the possible adrenergic involvement in the slow i.p.s.p.

Again the results of this study were quite clear. Concentrations of yohimbine (100 nM) 16 x's the IC_{50} for antagonism of the Ad_H failed to cause any reduction in the amplitude of the MCh_H or slow i.p.s.p. (see Fig. 29). Caution should be exercised when interpreting the results of experiments using high concentrations of α-antagonists. The antagonism of the slow i.p.s.p. by high doses of dihydroergotamine (40 μM) and phentolamine (200 μM) observed by Libet and Kobayashi (1974) may reflect non-specific inhibition of synaptic transmission (Yavari and Weight, 1981). It has been shown that high concentrations of α-antagonists may block Ca^{2+} channels in cultured neuroblastoma-glioma hybrid cells (Atlas and Adler, 1981). On the basis of the experiments in the present study it would seem that the slow i.p.s.p. does not involve an adrenergic component since direct responses to adrenaline can be blocked by concentrations of specific adrenergic antagonists (100 nM yohimbine) which have no effect on the MCh_H or slow i.p.s.p. In another series of experiments (Smith, unpublished observations) the slow i.p.s.p. in bullfrog sympathetic neurones has been found to be insensitive to 500 nM idazoxan.

4. Effects of cadmium on the slow i.p.s.p. and MCh_H

Prolonged exposure of an isolated ganglion to Ca^{2+}-free or Ca^{2+}-free high Ca^{2+}-blocker (eg.'s Ca^{2+}, Mg^{2+}, Mn^{2+}) Ringer's solutions may have deleterious effects on membrane responsiveness (Weight and Smith, 1980). Note for example that the Ad_H recorded by the sucrose-gap technique in Rana pipiens sympathetic ganglia declines in a Ca^{2+} free Ringer's solution, but is unchanged following the same period of exposure (25 min) to 100 μM Cd^{2+} (normal Ca^{2+}) Ringer's solution. In order to avoid possible non-specific membrane effects of Ca^{2+}-free Ringer's solutions and the commonly used "Ca^{2+}-blocking" Ringer's solutions (Weight and Padjen, 1973b; Weight and Smith, 1980; Horn and Dodd, 1981), a low concentration of the inorganic Ca^{2+}-blocker, Cd^{2+} (100 μM), was used to examine the possibility that the MCh_H involves excitation-secretion coupling for the release of a catecholamine from an interneurone. While synaptic transmission (slow i.p.s.p.) was completely abolished within 15 minutes, the MCh_H was conversely potentiated in amplitude (see Fig. 31). These results would seem to support a direct muscarinic action without any involvement of an adrenergic interneurone.

5. Concluding remarks

The results of the three experimental paradigms discussed above would lend support to the hypothesis of Weight and his colleagues (Weight and Padjen, 1973b; see Section A4d of the Introduction) suggesting that the slow i.p.s.p. in frog sympathetic ganglia occurs via a direct monosynaptic action of ACh on inhibitory muscarinic receptors. The disynaptic, adrenergic mechanism formalized by Libet (1970) has received extensive criticism from investigators working with both amphibian (Horn and Dodd, 1981; Weight, 1983; Horn and Dodd, 1983; Dodd and Horn, 1983b) and mammalian ganglion preparations (Dun, 1980; Cole and Shinnick-Gallagher, 1980; 1984). Many of the specific arguments have been presented in Section A4d of the Introduction (see also Libet, 1985; Shinnick-Gallagher and Cole, 1985). The general problem with the Libet hypothesis is that it was formulated on the basis of results obtained with pharmacological agents which have since (early 1960's)proven to be non-specific or inappropriate by comparison to those now available.

E. Summary

1) Hyperpolarizations to superfused adrenaline (Ad_H) could be recorded by the sucrose-gap technique from Rana pipiens and Rana catesbeiana sympathetic ganglia (EC_{50} = 1.65 μM; E_{max} = 0.5 - 3 mV). Despite this, an Ad_H was not recorded intracellularly from either B or C cells of Rana catesbeiana sympathetic ganglia. This discrepancy may be explained in terms of differences in the two recording techniques.

2) The Ad_H to submaximal concentrations of Ad recorded from Rana pipiens sympathetic ganglia can be potentiated by the catecholamine uptake blocker, desmethylimipramine (DMI). The log-concentration effect curve to Ad in the presence of 500 nM DMI was shifted in a parallel fashion to the left (EC_{50} = 270 nM). This may suggest that a catecholamine uptake system exists in amphibian sympathetic ganglia.

3) Based on studies with a variety of adrenergic agonists and antagonists, the Ad_H in Rana pipiens appeared to be mediated by an α_2-adrenoceptor. This conclusion was particularly supported by the observations that the α_2-adrenoceptor antagonist yohimbine antagonized the Ad_H with high affinity (IC_{50} = 6.2 nM).

4) Despite the existence of an α_2-adrenoceptor mediated hyperpolarization and an apparent DMI-sensitive catecholamine uptake mechanism in Rana pipiens sympathetic ganglia; studies with DMI, yohimbine and cadmium on the slow i.p.s.p., MCh_H and Ad_H did not

support a catecholaminergic involvement in slow synaptic inhibition in this ganglion. The results of this study reinforce the contention that the slow i.p.s.p. in amphibian sympathetic ganglia is generated by an inhibitory action of ACh on muscarinic receptors situated on the primary postganglionic neurones.

5) The possibility that electrogenic NaK-pump stimulation underlies the Ad_H was examined using the NaK-pump inhibitor, orthovanadate (Na_3VO_4). Although Na_3VO_4 was found to inhibit NaK-ATPase activity isolated from bovine brain (IC_{50} = 690 nM) and bullfrog CNS tissue (IC_{50} = 3.0 μM), 1 mM Na_3VO_4 had no inhibitory effect on the electrogenic NaK-pump responses, the ACh_{AH} and the K_H. It was concluded that orthovanadate was an unsatisfactory tool for studying the electrogenic NaK-pump *in situ* since it did not appear to gain access to its cytoplasmic site of action in these neurones.

6) Paradoxically, high concentrations (100 μM - 1 mM) of externally applied Na_3VO_4 did rapidly and reversibly antagonize the Ad_H presumably by an extracellular mechanism. It was speculated that this effect might involve stimulation of adenylate cyclase activity similar to that observed in cardiac muscle.

7) The Ad_H was blocked by 2 mM Ba^{2+} which supports the suggestion that it may be generated by an increase in gK. Since the response was not antagonized by Cd^{2+} (100 μM) or Mn^{2+} (10 mM), Ca^{2+} influx would not appear to be required for activation of the gK

underlying the Ad_H

8) An examination of the possible involvement of intracellular Ca^{2+} release in the α_2-adrenoceptor mediated increase in gK (Ad_H) was made by comparing the effects of various agents on the Ad_H (recorded by the sucrose-gap technique) with $Caff_H$'s (recorded intracellularly from B cells). It was found that none of the agents tested reliably affected the Ad_H and $Caff_H$'s in identical manners. In conclusion, this study did not reveal an obvious requirement for intracellular Ca^{2+} release for the electrogenesis of the Ad_H.

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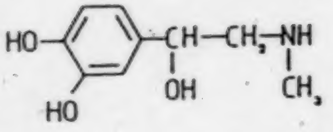
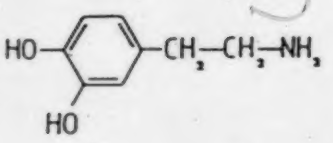
APPENDIX

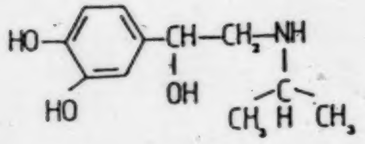
ADRENERGIC AGONISTS



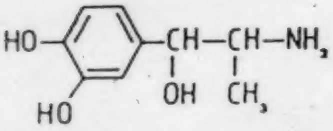
adrenaline bitartrate
(Sigma)



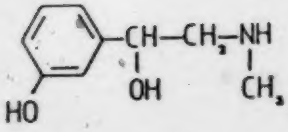
dopamine hydrochloride
(Nutritional Biochem.Corp)



isoprenaline bitartrate
(Sigma)

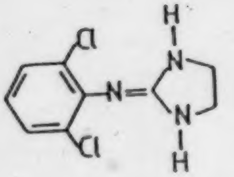


α-methylnoradrenaline hydrochloride
(Sterling-Winthrop.Res.Ltd)

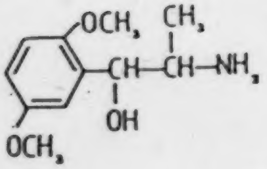


phenylephrine hydrochloride
(Sigma)

ADRENERGIC AGONISTS - continued

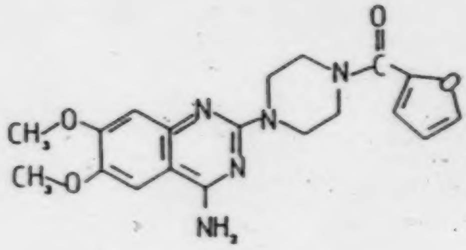


clonidine hydrochloride
(Boehringer Ingelheim)

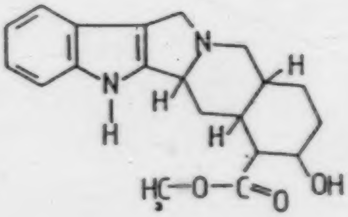


methoxamine hydrochloride
(Burroughs Wellcome Ltd.)

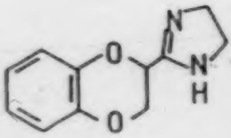
ADRENERGIC ANTAGONISTS



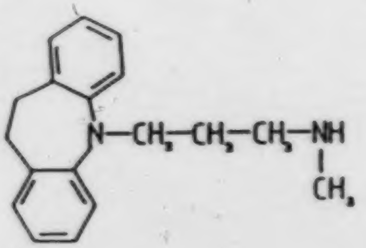
prazosin hydrochloride
(Pfizer)



yohimbine hydrochloride
(Sigma)

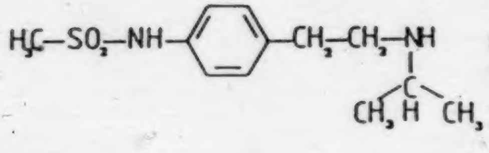


idazoxan (RX781094)
(Reckitt&Colman)

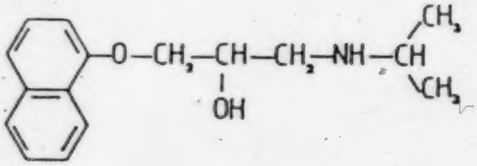


desmethylimipramine hydrochloride
(Ciba-Geigy.Pharm)

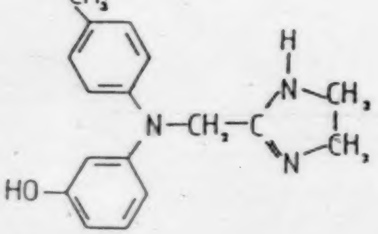
ADRENERGIC ANTAGONISTS



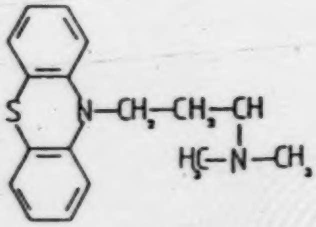
sotalol hydrochloride
(Bristol-Myers.Co)



propranolol hydrochloride
(Sigma)

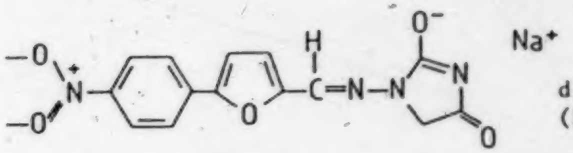


phentolamine hydrochloride
(Ciba)

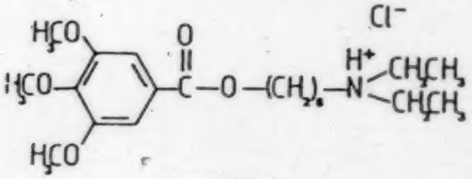


chlorpromazine hydrochloride
(Sigma)

INTRACELLULAR CALCIUM ANTAGONISTS

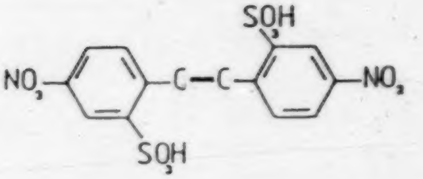


dantrolene sodium
(Norwich Eaton Pharm.Ltd)

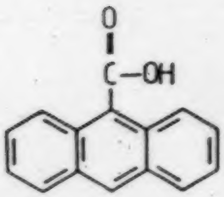


N,N-diethylamino(octyl)-
3,4,5,trimethoxybenzoate
(Aldrich)

ANION CHANNEL BLOCKERS

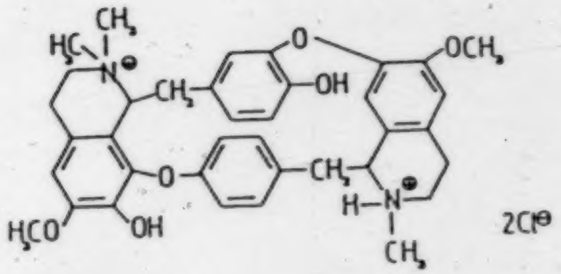


dinitrostilbene disulphonic
acid
(Aldrich)

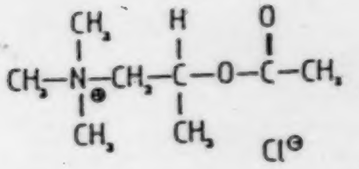


anthracene-9-carboxylic
acid
(Aldrich)

CHOLINERGIC AGENTS

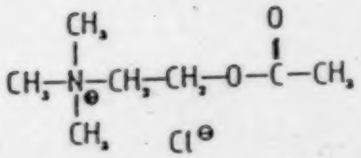


d-tubocurarine chloride
(Sigma)



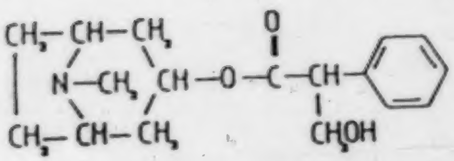
$$CH_3-\overset{CH_3}{\underset{CH_3}{N^{\oplus}}}-CH_2-\overset{H}{\underset{CH_3}{C}}-O-\overset{O}{C}-CH_3 \quad Cl^{\ominus}$$

methacholine chloride
(Sigma)



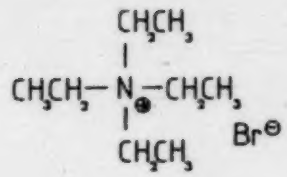
$$CH_3-\overset{CH_3}{\underset{CH_3}{N^{\oplus}}}-CH_2-CH_2-O-\overset{O}{C}-CH_3 \quad Cl^{\ominus}$$

acethylcholine chloride
(Sigma)



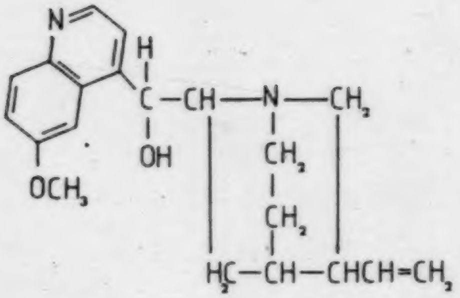
atropine sulphate
(Sigma)

POTASSIUM CHANNEL BLOCKERS

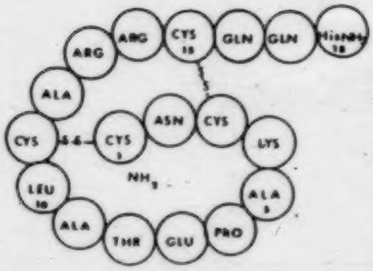


$CH_3CH_2-\overset{CH_2CH_3}{\underset{CH_2CH_3}{N^{\oplus}}}-CH_2CH_3 \quad Br^{\ominus}$

tetraethylammonium bromide
(Sigma)



quinidine hydrochloride
(Sigma)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ARG
CYS 15
GLN
GLN
His-NH₂ 18
ALA
CYS
S-S
CYS 3
ASN
CYS
LYS
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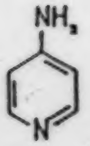
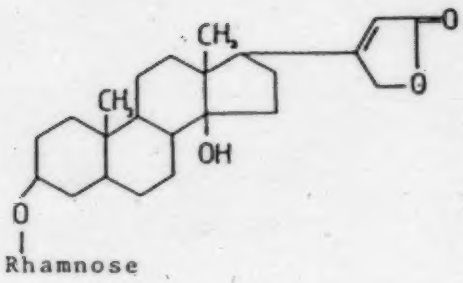
ALA
THR
GLU
PRO

apamin
(Sigma)



4-aminopyridine
(Sigma)

MISCELLANEOUS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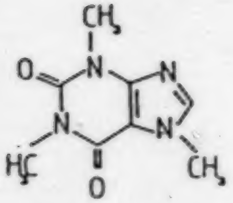
CH₃
OH
O
Rhamnose
O
O

ouabain - cardiac glycoside
(Sigma)


CH₃
O
N
N
N
N
H₃C
O
CH₃

caffeine - methyl xanthine
(Sigma)